Genes expressed in pollinated pistils of *Solanum tuberosum*

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OF *SOLANUM TUBEROSUM*

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
woensdag 27 november 1996
des namiddags om 3.30 uur precies

door

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geboren op 11 november 1966 te Oosterhout (Gld.)

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The studies described in this thesis were financially supported by the European Community Bridge programme (BIOT 900172)

voor mijn ouders

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Ik ween om bloemen in den knop gebroken,
en vóór den uchtend van haar bloei vergaan.

naar W. Kloos

CHAPTER 1

Introduction to sexual plant reproduction: the role of the pistil during pollen tube growth

Introduction

Sexual reproduction is a key process in the life cycle of the plant and is of crucial importance for the diversity and preservation of plant species. It is also the basic process for the use of plants for food production and ornamental flowers. In seed plants, sexual reproduction occurs in a specialised structure, the flower, consisting of sterile and reproductive organs. The sterile parts of the flower are the sepals and the petals, whereas the stamens and carpels form the reproductive parts (Esau, 1977).

Flower formation

The first step in the process of sexual reproduction in seed plants is the transition from vegetative to generative growth. This developmental change starts with floral initiation and is controlled by environmental stimuli, as light (photoperiod), temperature and water availability that act on an endogenous genetic programme. Floral initiation is followed by floral morphogenesis, which is characterised by the development of the different flower organs. During these processes, different sets of floral meristem- and organ-identity genes are expressed which regulate the transition from shoot to floral meristem and the control of flower organ identity (Coen and Meyerowitz, 1991; Huala and Sussex, 1993; Okamuro *et al.*, 1993; Coen and Carpenter, 1993).

Male reproductive organ

Within the flower, the stamen is the organ in which the male reproductive process takes place. It consists of the anther and the filament, which is a stalk containing vascular tissue that serves as a conduit for water and nutrients from the rest of the flower to the anther. The actual reproductive tissue of the anther is a group of sporogenous initial cells that give rise to pollen mother cells. The pollen mother cells undergo meiosis resulting in tetrads which each fall apart in four microspores. The microspores, in the locule of the anther, divide mitotically and eventually differentiate into bi- or tri-cellular male gametophytes, or pollen grains, that contain a vegetative and one or two generative cells (Goldberg *et al.*, 1993). The non-reproductive tissues of the anther originate from parietal initial cells, and are involved in the maintenance of

anther structure, and produce compounds necessary for male gametophyte development These compounds are mainly synthesised in the tapetum and deposited onto or taken up by the developing pollen At maturity the pollen are released from the dehiscing anther and are fully prepared for transport to the female reproductive organ, in which pollen germination and tube growth takes place

Female reproductive organ

The female reproductive organ in angiosperms is the gynoecium, which consists of one or more carpels At the mature stage of the flower, one carpel can be considered to consist of three parts the ovary, at the base of the carpel, containing the ovules, the style, an extension above the ovary, through which the pollen tubes grow towards the ovules, and the stigma, on top of the style, where pollen grains adhere, hydrate and germinate (Esau, 1977) Sometimes, the term pistil is used for the description of the gynoecium However, in this thesis the term pistil is used for the combination of only stigma and style

The development of the carpel is under control of organ-identity genes and is morphologically distinguishable by the formation of the carpel primordia Floral organ development is initiated by the divisions of the three cell layers designated as L1, L2, and L3 In the carpel, the L3 layer divides in various planes at the centre of the floral meristem (Satina, 1944) To obtain a closed carpel, either the margins of one carpel are fused resulting in a simple closed carpel, or a group of carpels are fused together to form a compound carpel Enclosed within the ovary, the ovules are developing which consists of nucellus, integuments and supporting stalk, the funiculus In the centre of the nucellus tissue, a single cell differentiates into a megasporocyte, which undergoes meiosis to produce four haploid megaspores The embryo sac (megagametophyte) then develops mostly from one of the megaspores The nucellus itself is enclosed by one or two integuments, which leave a small pore, the micropyle, through which the pollen tube enters the ovule After fertilisation, the ovules develop into seeds, the embryo sac gives rise to the embryo and endosperm, and the integuments differentiate into the seed coat

At the moment the ovary is closing, the tissue at the top of the ovary begins to extend vertically to form a pistil This extension is achieved by both cell division and cell elongation

The stigmatic secretory zone and the transmitting tract of the stylar canal are developed from the L1 layer, which is epidermal in origin. The central core of transmitting tract is surrounded by a cortex of parenchymatous cells (L2 layer origin), in which the vascular bundles are located which are formed by the L3 layer (Satina, 1944). The stylar cortex is enveloped in a non-secretory epidermis. The purpose of the vertical extension of the ovary is to facilitate pollination. The variety in style and stigma morphology is a reflection of the different pollination strategies found among the angiosperms. A style can be hollow (*Lilium*), the transmitting tract consists of a cavity (canal) surrounded by canal cells which deposit secretion products; semi-solid (*Cactaceae*), a small canal below the stigma and solid tissue near the ovary; or solid (*Petunia* and *Nicotiana*) in which the transmitting tissue consists of elongated cells organised into vertical files. The cells in the transmitting tract are very active in producing secretion products and are rich in organelles such as dictyosomes and endoplasmic reticulum (Knox, 1984). The surface of the stigma can either be wet (wet stigma) which means that at maturity it is completely covered with a fluid secretion (exudate), or dry in which state it lacks any form of secretion (dry stigma). The exudate is excreted late during pistil development by the stigmatic surface cells, whereas the exudate produced by the transmitting tissue cells fills the regions between the secreting cells. This mucilage provides a suitable environment for pollen germination and tube growth (Esau, 1977; Knox, 1984).

Pollen germination and pollen tube growth

After the pollen grains are released from the mature anther, they are transported to a receptive pistil by either insects or wind. The pollen grains adhere to the stigma by the sticky exudate of the wet stigma or, in case of a dry stigma, the sticky layer of the pollen itself (pollen coat). As the pollen grains germinate, compounds and water are transported from the stigma to the dehydrated pollen, which in case of a dry stigma is facilitated by the pollen coat (Ruiter, 1996). After pollen hydration, proteins (including hydrolytic enzymes) in the pollen or at the pollen coat are released at the site of the pore, the place where finally the pollen tube will protrude. When the pollen tube is formed, it penetrates the stigmatic layers and elongates through the stigma to the transmitting tissue of the style all the way down to the ovary, where it enters the ovules and discharges the two sperm cells. In the ovule, one of the sperm cells fuses with the

haploid egg cell to form the embryo, while the second sperm cell fuses with the central cell to form the polyploid endosperm.

The pollen tube synthesises its own components but the growth of the pollen tube is generally believed to be promoted by the pistil, since *in vitro* pollen tube growth is often slower and the length of the tubes shorter as compared to the *in vivo* growing pollen tubes. Furthermore, *in vitro* growing pollen tubes lack a targeted directionality (Mascarenhas, 1993; Cheung, 1995). During pollen tube growth low molecular weight components are taken up from the pistil and metabolised (Chen and Loewus, 1977; Deshusses *et al.*, 1981; Schlüpmann *et al.*, 1994; Capkova *et al.*, 1983; Vogt and Taylor, 1995; Vogt *et al.*, 1994). Among the low molecular weight substances taken up by the pollen or pollen tube are flavonols, a special class of flavonoids, present in both pistil and pollen (Koes *et al.*, 1994). The flavonols enhance development and germination of *in vitro* cultured pollen (Ylstra, 1995). Pollen of *Petunia* and maize mutants without flavonols are unable to form functional pollen tubes and to set seed (Pollak *et al.*, 1995; Ylstra, 1995). However, flavonol-deficient *Arabidopsis* tt4 mutants exhibit normal pollen tube growth and seed set (Ylstra, 1995; Bubulis *et al.*, 1996). The function of the flavonols during pollen development and pollen tube growth is still under investigation (see chapter 5). The process of promotion of pollen tube growth by the pistil takes, in general, only place when the pollen or pollen tubes are recognised by the pistil.

Recognition during pollen germination and pollen tube growth

After landing of the pollen on the stigma, the pistil is able to discriminate between the different types of pollen it receives and determines whether the pollen will be accepted or rejected (Knox, 1984). The pollen-pistil recognition leading to rejection of pollen or pollen tube can either be inter-specific (incongruity) or intra-specific (self-incompatibility). According to the species, pollen rejection can take place at three distinct moments after pollination: when the pollen has landed on the stigma, when the pollen tubes are growing within the transmitting tissue or when the pollen tubes have reached the ovary.

Self-incompatibility (SI) is a physiological mechanism with a genetic basis steered by the S-locus system with multiple alleles, and it promotes outbreeding (Linskens, 1981). On the S-locus, genes are localised responsible for products present in both pollen and pistil, both

involved in the SI reaction At the moment, only the proteins encoded by these S-genes expressed in the pistil have been described, and not the S-locus genes encoding proteins involved in the SI reaction at the pollen side

Within the homomorphic self-incompatibility system two different systems can be distinguished, sporophytic SI and gametophytic SI In the sporophytic SI systems, the pollen grains display the parental (sporophytic) phenotype at the stigma rather then expressing their own haploid (gametophytic) genotype This is manifested by the inability of the pollen to germinate and/or the inability of the emerging pollen tube to invade the stigma (Nasrallah and Nasrallah, 1993) The sporophytic SI system in *Brassicaceae* is characterised by two S-locus linked genes, encoding a S-locus glycoprotein (SLG) and a S-locus receptor kinase (SRK), the latter one showing extensive sequence homology in its extracellular domain with SLG (Nasrallah *et al*, 1988, Dwyer *et al*, 1989) Both types of S-genes show multi-allelic variation Though SLG and SRK genes are expressed in both pistil and anther, SLG genes in the pistil are developmentally expressed, and their expression pattern correlates with the onset of the self-incompatibility reaction (Nasrallah *et al*, 1988)

In gametophytic SI systems, rejection of pollen tubes in general does not take place at the stigma, but within the style It occurs when the S-allele of the haploid pollen matches either of the alleles in the pistil (Newbigin *et al*, 1993) However, there are some species (*Papaver rhoeas*) which possess an intermediate SI system with a gametophytic determination but with rejection of the pollen on the stigmatic surface In solanaceous plants the S-genes encoded glycoproteins are directly involved in the SI reaction and the localisation coincides with the path that the pollen tubes follow as they grow through the pistil (Newbigin *et al*, 1993, Lee *et al*, 1994, Murfett *et al*, 1994) The S-glycoproteins, which display ribonuclease activity (S-RNases), are undoubtely linked to the SI reaction (McClure *et al*, 1989, Huang *et al*, 1994, Royo *et al*, 1994) and are taken up by pollen tubes where they degrade pollen tube rRNA (Gray *et al*, 1991, McClure *et al*, 1990) In *Nicotiana alata* pistils the S-RNases are phosphorylated by Ca^{2+}-dependent protein kinases from pollen tubes (Kunz *et al*, 1996) In *Papaver rhoeas* the S-glycoproteins are also phosphorylated, but display no ribonuclease activity which indicates that gametophytic SI is also possible without RNases

Guidance of the pollen tubes

For guidance of pollen tubes two mechanisms can be distinguished. The growing pollen tubes can be either guided physically, by the morphological structure of the stigma, style and ovary, and/or chemotropically by compounds produced in these tissues.

The physical guidance is observed in pearl millet where the trichomes on the stigma direct the pollen tubes to the style and ovary (Heslop-Harrisson and Reger, 1988). In maize, the pollen germinating on a silk hair is directed to the transmitting tissue by the orientation of the receptive silk hair (Bedinger *et al.*, 1994). In spinach, the direction of pollen tube growth in the transmitting tissue of the style is determined by the morphology and distribution of the central core of the transmitting tissue and by the structure of the cell walls (Wilms, 1980). The physical guidance hypothesis is further strengthened by experiments which showed that latex beads are translocated through the transmitting tissue to the ovaries at rates similar to those of growing pollen tubes (Sanders and Lord, 1989; Lord and Sanders, 1992). *Arabidopsis* mutants defective in ovule development show disorganised pollen tube growth in the ovary, indicating that the ovules are important for pollen tube guidance (Hülskamp *et al.*, 1995).

Proof for chemotropical guidance of pollen tubes comes mostly from experiments *in vitro* (Derksen *et al.*, 1995). The pollen tubes from *Antirrhinum* and pearl millet are attracted *in vitro* by Ca^{2+} and glucose (Mascarenhas and Machlis, 1962b; Reger *et al.*, 1992b). But also the extracts of different pistil tissues or proteins from the transmitting tissue have been shown to attract pollen tubes *in vitro* or semi-*in vivo* (Mascarenhas, 1975; Mascarenhas and Machlis, 1962a; Cheung *et al.*, 1995; Wu *et al.*, 1995). In ovaries, pollen tubes are attracted by exudate from the ovules which is rich in carbohydrates and a high concentration of Ca^{2+} is present in the synergid cells which could be important in pollen tube attraction (Franssen-Verheijen and Willemse, 1993; Chaubal and Reger, 1993; Reger *et al.*, 1992a).

Nourishment of growing pollen tubes

The pistil not only guides the pollen tube, but it also produces nourishing compounds for the growing pollen tubes. The stigmatic exudate is, in most species with a wet stigma, lipidic in nature (Knox, 1984), but also carbohydrates, (glyco)proteins and sometimes phenolic compounds are present (Konar and Linskens, 1966a, 1966b; Kandasamy and Kristen, 1987;

Labarca *et al.*, 1970; Mackenzie *et al.*, 1990; Cresti *et al.*, 1986; Gleeson and Clarke, 1979; Du *et al.*, 1996). Carbohydrates in the stigmatic exudate of *Lilium longiflorum* are taken up by the germinating pollen and are metabolised (Labarca and Loewus, 1972). Transgenic plants with pistils lacking a stigmatic secretory zone are female-sterile, because pollen tubes are unable to penetrate the transmitting tissue. Fertility can be restored by application of stigmatic exudate of wild-type pistils (Goldman *et al.*, 1994). These experiments indicate that the products produced by the stigmatic secretory zone are necessary for pollen tube growth and that the nutritional or guiding role of the pistil already starts at the first moment of contact between the pollen and stigma.

The cells of the transmitting tissue excrete an extracellular matrix which is, just as the stigmatic exudate, rich in sugars, free amino acids (Tupy, 1961; Kovaleva and Komarova, 1993) and fatty acids (Cresti *et al.*, 1986), and many of these compounds are part of more complex molecules such as polysaccharides, glycolipids and glycoproteins (Atkinson *et al.*, 1994). The major class of proteins detected in the extracellular matrix of the transmitting tissue is the hydroxyproline-rich arabinogalactan (AG) proteins (Bacic *et al.*, 1988; Sedgley *et al.*, 1985). The carbohydrate side-chains are composed of a branched galactan framework, substituted primarily with arabinose residues but also with rhamnose, mannose and xylose (Fincher *et al.*, 1983). In *N. alata,* some AG or AG-like proteins from the pistil have been purified, and the encoding mRNAs isolated (Du *et al.*, 1994; Lind *et al.*, 1994). These AG proteins are supposed to provide nutrients for pollen tubes in their heterotrophic phase of growth (Fincher *et al.*, 1983; Labarca and Loewus, 1972; Labarca and Loewus, 1973) and the uptake by pollen tubes of a 120 kDa AG protein of *N. alata* has been demonstrated (Lind *et al.*, 1996).

A diverse group of other proline-rich sequences, specifically expressed in the pistil, with a assumed role in the nourishment of growing pollen tubes has been isolated (Wang *et al.*, 1993; Chen *et al.*, 1993; Cheung *et al.*, 1993). Among them is a special group of proline-rich sequences characteristic for extensin-like proteins (Baldwin *et al.*, 1992; Chen *et al.*, 1992; Wu *et al.*, 1993; Goldman *et al.*, 1992). One of the transmitting tissue-specific proline-rich proteins, TTS of tobacco, promotes pollen tube growth *in vitro* and *in vivo*, and attracts pollen tubes grown in a semi-*in vivo* culture system (Cheung *et al.*, 1995, Wu *et al.*, 1995). This

highly glycosylated TTS protein is deglycosylated upon pollination and the protein backbone is incorporated into the pollen tube wall (Wang *et al.*, 1993; Wu *et al.*, 1995). The sugar moieties on the TTS proteins probably are taken up by the pollen tubes as a source of nutrients. The pollen tubes need to produce molecules which enhance the uptake of nutrients, such as monosaccharide transporters (Ylstra, 1995).

Protection of the pistil upon pollination

The physical penetration of non-aseptic pollen tubes in the pistil implies wounding and protection against pathogens is thus required. The vulnerability of the pistil for pathogens before pollination could be due to the large spaces between the cells of the stigma and transmitting tissue and is further enhanced by the breakdown of the physical barrier by the penetrating pollen tubes. The observation that rarely any pathogen infection is introduced via the pistil confirms the presence of an active defence mechanism in pistils (Jung, 1956). The production of defence substances, toxic for pathogens, is expected during the penetration of the pistil by the growing pollen tubes. The presence of such molecules before pollination would provide an even more effective defence barrier. In relation to this function, a set of various pistil-expressed genes with a defence-related function have been described.

Thionins, a group of proteins implicated in plant defence against pathogens (Florack and Stiekema, 1994), have been isolated from pistil tissues. Especially the γ-thionins have been detected in pistils of tobacco, *Petunia*, tomato and potato (Gu *et al.*, 1992; Karunanandaa *et al.*, 1994; Milligan and Gasser, 1995; see chapter 4). Within the carpel of tobacco, floral-specific thionin (FST) transcripts were observed in the cortex of the style, the carpel wall and around the ovules. Transcription was inducible in sepals and leaves by fungal pathogen infection (Gu *et al.*, 1992).

From *N. alata*, a stigma-specific precursor of a proteinase inhibitor was isolated which *in vivo* produces five similar proteinase inhibitor peptides by proteolytic processing (Atkinson *et al.*, 1993). This proteinase inhibitor may have a defence role in the stigma because similar proteinase inhibitors are effective against proteases of fungal, bacterial and insect origin (Ryan, 1990).

The various hydroxyproline-rich glycoproteins (HRGP) present in pistils of several species

(Sommer-Knudsen *et al*, 1996, Wang *et al*, 1993, Chen *et al*, 1993, Cheung *et al*, 1993) do not only play a role in nutrition of the pollen tube, but may be involved in the defence against pathogen attack by immobilising invading bacterial cells at the cell wall The protein backbone of some HRGPs has similarities to the agglutinins from potato and tobacco which have the ability to agglutinate cells of potentially pathogenic bacteria (Mellon and Helgeson, 1982, Leach *et al*, 1982) Wounding of pistils resulted in a marked increase in expression of an hydroxy-proline rich extensin gene isolated from carrot (Chen *et al*, 1992)

Different kinds of pathogenesis-related (PR) proteins and mRNAs are demonstrated to be present in the pistil tissues Pathogenesis-related chitinase activity was found in the ovary of tobacco, in the stigma of *Petunia* and in the style of tomato and potato (Budelier *et al*, 1990, Gasser *et al*, 1989, Lotan *et al*, 1989, Leung, 1992, Harikrishna *et al*, 1996, Wemmer *et al*, 1994) The (1,3)-β-glucanases identified in the extracellular matrix of the transmitting tissue of tobacco pistils are also supposed to be involved in protecting the style, and it was demonstrated that these proteins were not essential for reproduction in tobacco (Lotan *et al*, 1989, Ori *et al*, 1990, Sessa and Fluhr, 1995, Côte *et al*, 1991) The expression of a PR-10a gene from potato can be induced in various parts of the plant by *Phytophthora infestans*, whereas in healthy, unstressed plants PR-10a is exclusively expressed in the papillae and uppermost cell layers of the stigma (Constabel and Brisson, 1995) Furthermore, a potato pistil-specific gene homologous to PR-1 is expressed in both stigma and stylar cortex (chapter 3, Van Eldik *et al*, 1996) PR-1 proteins can be detected in styles of tobacco pretreated with a pathogenesis elicitor (Lotan *et al*, 1989) Taken together, some genes of which expression is normally induced after wounding or pathogen attack, are expressed in healthy plants in various parts of the pistil before and after pollination These defence-related proteins could probably be involved in processes other than protection in the pistil

Outline of this thesis

This thesis describes research on the interactions between the pistil and growing pollen tube The view taken is, that the pistil is involved in recognition, guidance, nourishment and protection of the growing pollen tubes In order to collect more molecular knowledge on the various functions of the pistil, the transcripts of genes and gene products accumulating in the

pistil in response to growing pollen tubes were studied The investigations reported were therefore aimed at studying the pollinated pistil of *Solanum tuberosum* to obtain an over-all picture of the transcripts and gene products involved in the various functions of the pistil during pollen tube growth

In chapter 2 the sts15 cDNA clone is described which was isolated after differential screening of a pollinated pistil cDNA library Two cDNA clones are isolated which are similar to γ-thionin-like and PR-1 genes, both encoding proteins involved in plant defence (chapter 3 and 4) The isolated alcohol dehydrogenase(ADH)-like clone is possibly involved in nourishment of the pollen tubes, moreover ADH activity and *adh* gene expression were induced upon pollination (chapter 4) Regarding the pollen tube growth promoting function, a flavonol synthase clone was isolated, encoding an enzyme involved in the synthesis of the pollen tube growth promoting flavonols (chapter 6) Furthermore, several clones were isolated, without homology to known sequences, of which the roles in the various functions of the pistil is not clear (chapter 2, 4 and 5)

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CHAPTER 2

A pistil-specific gene of *Solanum tuberosum* is predominantly expressed in the stylar cortex

published in *Sexual Plant Reproduction* (1995) 8: 173-179

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Abstract

In a programme aimed at studying genes expressed in pistils, the cDNA clone sts15 was isolated from a cDNA library of pollinated pistils of *Solanum tuberosum* and was found to be expressed only in pistils. During development of the pistil, the accumulation of sts15 transcripts, which are 0.7 kb long, reached a maximum just before anthesis and declined in fully open flowers. Southern blot analysis revealed that *sts15* was present as a small gene family in dihaploid potato. *In situ* hybridisation experiments indicated that *sts15* was strongly expressed in the cortex of the style and at a low level in the stigma. No hybridisation signal was observed in the transmitting tissue. The temporal and spatial expression patterns of *sts15* indicate that the gene products of the *sts15* gene might be involved in the function of the stylar cortex or in making the pistil prepare for pollination.

Introduction

In flowering plants, the pollen, the male gametophytes, and the pistil, which contains the female gametophytes (Esau, 1977), come into close contact at the onset of pollination. During this interaction, the genetic background of both tissues determines whether or not successful fertilisation will take place Although rejection of the pollen, or self-incompatibility, is extensively investigated at the molecular level (Sims, 1993), relatively few data have been obtained that shed light on the molecular basis of a successful pollen-pistil interaction. Such an interaction is characterised by germination and undisturbed growth of the pollen tubes that ultimately leads to fertilisation of the egg cell in the embryo sac (Lord and Sanders, 1992)

The process of pollination starts when mature pollen, developed in the pollen sacs of the anther (Mascarenhas, 1990), is shed and transferred to the mature stigma of a receptive pistil After landing, recognition processes initiate the cascade of events that lead to fertilisation (Mascarenhas, 1990) This recognition implies that pistils are able to discriminate between the

different types of pollen they receive and to determine whether they will accept or reject the pollen (Knox, 1984). According to the species, pollen acceptance or rejection may take place at two distinct moments after pollination, either when the pollen has landed on the stigma or when the pollen tubes are in the style. After the pollen has been accepted on the stigma, it germinates and produces a pollen tube. This pollen tube penetrates the stigmatic tissue (Van Went and Willemse, 1984) and grows into the intercellular matrix of the stylar transmitting tissue. In some species, the choice between acceptance and rejection is delayed until the pollen tube has travelled one third of the length of the style (gametophytic self-incompatibility, such as in the *Solanaceae*). After the pollen or the pollen tubes are accepted, it is believed that successful growth depends on the qualitative and quantitative composition of the nutrients in the stylar transmitting tissue. The intercellular matrix of the transmitting tissue is filled with proteinaceous substances and low-molecular-weight compounds like glucose and galactose (Herrero and Dickinson, 1979; Konar and Linskens, 1966; Sedgley *et al.*, 1985). Its mass is influenced before and during pollination by temperature and other environmental factors (Van Herpen, 1984). Compounds in the tissue may affect the growth of the pollen tubes (Herrero and Arbelo, 1989; Ichimura and Yamamoto, 1992; Kroh *et al.*, 1971), because substances from the intercellular matrix are taken up by the pollen tubes (Kroh *et al.*, 1970; Labarca and Loewus, 1973). In the *Solanaceae*, for example, the S-RNases are taken up *in vitro* by the pollen and pollen tube (Gray *et al.*, 1991) and determine whether the pollen tube growth will be arrested or will proceed (Lee *et al.*, 1994; Murfett et *al.*, 1994).

To understand more about the genes that are involved in the acceptance or rejection of the pollen and pollen tubes in the different tissues, several pistil genes have been isolated and are being investigated. These genes, predominantly expressed in the pistil, can be divided into two major groups (Gasser and Robinson-Beers, 1993). The first group contains the S-genes involved in self-incompatibility, these are all expressed in the pistil and thoroughly described (Nasrallah and Nasrallah, 1993; Newbigin *et al.*, 1993; Sims, 1993). The second group comprises pistil genes that are not involved in self-incompatibility. This group includes genes expressed in the stylar transmitting tissue of tomato (Budelier *et al.*, 1990; Gasser *et al.*, 1989) and tobacco (Ori *et al.*, 1990). Several genes in this group encode proline-rich proteins. Some of them are extensin-like (Baldwin *et al.*, 1992; Chen *et al.*, 1992; Goldman *et al.*, 1992),

whereas others are characterised only by a high proline content (Chen *et al.*, 1993; Cheung *et al.*, 1993). Other pistil-specific genes are a proteinase inhibitor precursor of *Nicotiana alata* (Atkinson *et al.*, 1993) and STIG1, a tobacco gene that is specifically expressed in the stigmatic secretory zone (Goldman *et al.*, 1994). None of these genes described so far have been identified as specific for compatible pollination; however, some extensin-like genes have a modulated expression upon pollination (Goldman *et al.*, 1992; Wang *et al.*, 1993; see also chapter 5 and 6). Also, nothing is known on the effect of the pollen gene products on the expression of these pistil-specific genes.

The aim of our research was the isolation and characterisation of genes expressed in pollinated pistils and the establishment of their functional role during pollen tube-pistil interaction. To achieve these goals, we differentially screened a cDNA library of pollinated pistils of *Solanum tuberosum* and isolated several pistil-specific cDNAs. The temporal and tissue-specific expression and localisation of the transcripts of one of these genes and its putative function are described.

Results

Identification of a gene predominantly expressed in pistils

To isolate genes expressed in pollinated pistils during compatible pollination, a cDNA library of cross-pollinated potato pistils was constructed and differentially screened against unpollinated pistils. Although we expected to identify pollination-induced genes, by this protocol we isolated two recombinant clones which proved to be only pistil-specific and were not pollination-induced. One of them, sts15, was chosen for further analyses.

The tissue-specific expression pattern of the *sts15* gene was determined by Northern blot hybridisations of total RNA of several tissues (Figure 1). The sts15 cDNA strongly hybridised to a single mRNA transcript of 0.7 kb present in pistils. At lower stringency, a very weak signal could be observed in ovaries and sepals; the signal was the same size as in pistils (data not shown). Sts15 transcripts were not detected in any other tissue even after extended

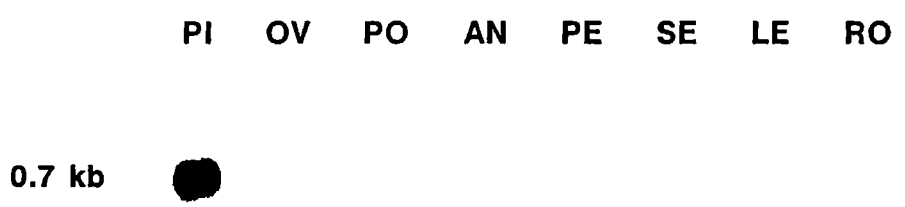

Figure 1 Tissue-specific expression of the pistil-specific *sts15* gene
Equal amounts of total RNA from different tissues were probed with ^{32}P-labelled sts15 cDNA. Following hybridisation the blot was washed in 1x SSC, 0.1% SDS at 55°C and used for autoradiography. The size of the RNA transcripts is indicated at the left. PI, pistil; OV, ovary; PO, pollen; AN, anther; PE, petal; SE, sepal; LE, leaf; RO, root.

exposure.

To detect sts15 transcripts during pistil development, RNA extracted at four stages (see Materials and Methods) of flower development (Kaufmann *et al.*, 1991) was probed with sts15 cDNA. The sts15 mRNA was already present at stage 2, then increased and reached a maximum at stage 3. At anthesis, the sts15 mRNA level had decreased (Figure 2).

Localisation of sts15 *spatial expression*

The expression of *sts15* in the different pistil tissues was localised by *in situ* hybridisation carried out on longitudinal sections of pistils at stage 3. Figure 3 shows representative results of the hybridisations. An overview of a complete pistil with bright-field illumination is given in Figure 3A. Using an antisense RNA probe of sts15 and detection of the hybridising signal with confocal laser scanning microscopy (CLSM), a very strong signal was detected in the cortex of the style (Figure 3B). Whether the sts15 mRNA is also present in the epidermis could not be distinguished with this approach (Figure 3B). No hybridisation signal was observed in the transmitting tissue, the stylar part of the pistil surrounded by the cortex (Figure 3C). The hybridisation level in the stigma was much lower than in the cortex of the style (Figure 3D). Occasionally, hybridisation was observed in the papillae. The sense sts15 cDNA probe showed

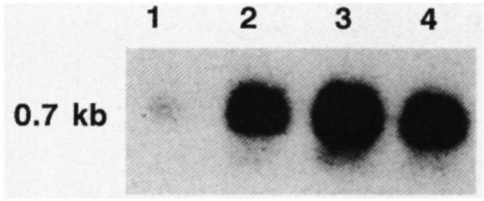

Figure 2 Expression of the *sts15* gene during pistil development

Lanes 1 to 4 correspond to the four stages of flower development (Kaufmann *et al.*, 1991). Lane 1, early green bud; lane 2, closed bud and anthers are turning yellow; lane 3, partly opened bud; lane 4, flower at anthesis. Total RNA was isolated from pistils of the different developing stages and equal amounts of RNA were probed with the sts15 cDNA. The size of the RNA transcripts is indicated at the left. Following hybridisation the blot was washed in 1x SSC, 0.1% SDS at 55°C and used for autoradiography.

no hybridisation in any tissue examined (Figure 3E and 3F).

Sequence analysis of sts15 cDNA

Sequence analysis of sts15 cDNA revealed one open reading frame and a deduced protein of 183 amino acids (Figure 4), resulting in a predicted molecular mass of 20.4 kDa. The deduced amino acid sequence is rich in leucine and serine, and does not contain any tryptophan. A hydrophobic putative signal peptide of 23 amino acids residues is predicted from the sts15 cDNA sequence, together with a potential cleavage site located between amino acid 23 and 24 (Von Heijne, 1986). Computer analysis also revealed a transmembrane helix located in the signal peptide from amino acid 2 to 20. The protein has a predicted high isoelectric point of 9.24. Four potential N-glycosylation sites are located on amino acids 15, 37, 75 and 96. The sts15 cDNA nucleotide and amino acid sequences do not display any homology with sequences in the computer databases.

Conservation of the sts15 *gene and its expression in other species*

The conservation of the *sts15* gene in other plant species was determined at DNA and RNA level (Figure 5). Genomic DNA was isolated from young leaves of *Brassica oleracea*, *Nicotiana tabacum* cv. Petit Havana, *Petunia hybrida* and *Solanum tuberosum*, and analysed by Southern hybridisation. The presence of two faint hybridising fragments in *B. oleracea*, one strong band in *P. hybrida* and two bands in *N. tabacum* (Figure 5A) indicated that the *sts15*

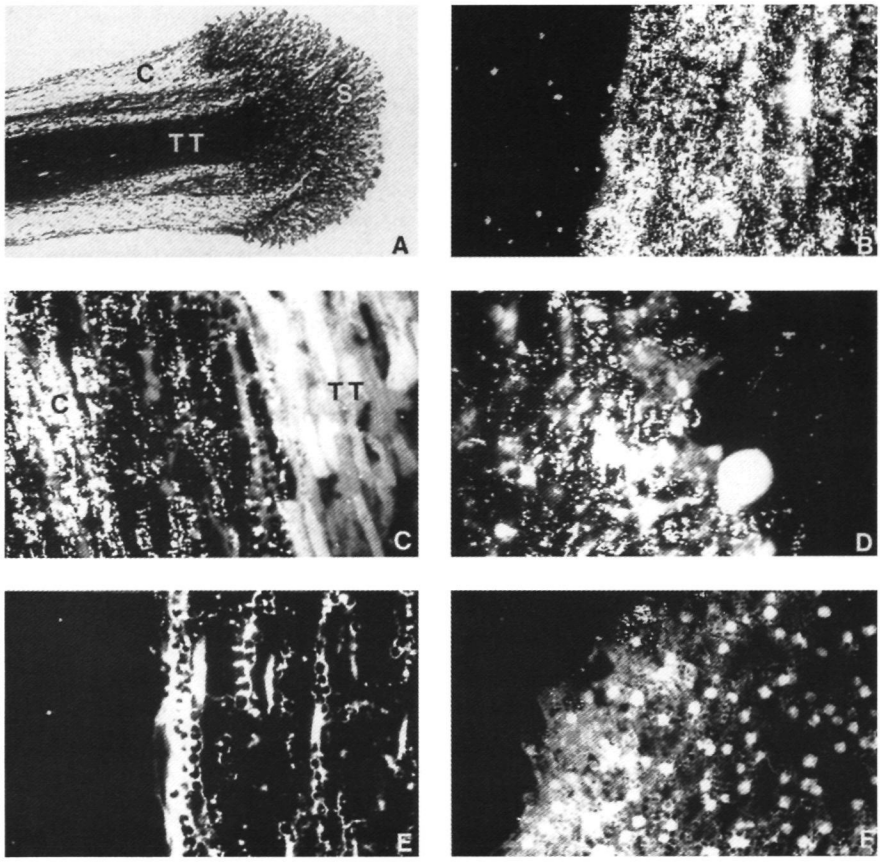

Figure 3 Spatial expression pattern of the *sts15* gene in potato pistils

A. Bright-field illumination of a longitudinal section of a potato pistil stained with toluidine blue, showing the stylar cortex (C), the transmitting tissue (TT) and the stigma (S). Magnification 20x. B-F. *In situ* hybridisation of sts15 in longitudinal sections stained with ethidium bromide. B Antisense sts15 probe hybridising with the stylar cortex. Magnification 520x. C. Antisense sts15 probe only hybridising in the cortex, no hybridisation signal in the transmitting tissue. Magnification 520x. D. Antisense sts15 probe hybridising with the stigma of the pistil. Magnification 520x. E. Sense sts15 probe. No hybridisation signal in the stylar cortex or epidermis. Magnification 360x. F. Sense sts15 probe. No hybridisation signal in the stigma. Ethidium bromide staining of the nuclei. Magnification 360x. Transmitting tissue (TT) and stylar cortex (C).

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  1  AAAAGGTCATGTCAATTGTCATACCAAAAACATGAAGCATCTTTCCTCTTTCTTCCTATT
  1                                  M  K  H  L  S  S  F  F  L  F

 61  TCTCTCTTTATTTAACCTAACATTCTATGGCTCAACAGCCCAAAATTTAATCCAAACAAC
 11   L  S  L  F  N  L  T  F  Y  G  S  T  A  Q  N  L  I  Q  T  T

121  TTGCAAATCATGTTCCAAAAATGAATCAAGTATTACGTATGGCTTTTGCACTTCATCTCT
 31   C  K  S  C  S  K  N  E  S  S  I  T  Y  G  F  C  T  S  S  L

181  ACAAGCTGCTCCGGCGAGTCAATGTGCTACTCTCCGTGGCCTTGGAATGATTTCTATTAG
 51   Q  A  A  P  A  S  Q  C  A  T  L  R  G  L  G  M  I  S  I  R

241  ATTAATTCGATACAATGTTACTGATACGAGGTGTCACGTTAAAATGTTGTTGAAGGACAA
 71   L  I  R  Y  N  V  T  D  T  R  C  H  V  K  M  L  L  K  D  K

301  GAATTTGGATCTTTATAATAGGAGTCGCTTGAAAGTTTGTTTAGATCTTTATTCTGACGC
 91   N  L  D  L  Y  N  R  S  R  L  K  V  C  L  D  L  Y  S  D  A

361  GATCCCTACTATCAAGCTTGCTATGAAGAGTTACAATACGAAGAAATATTATGATGCGAA
111   I  P  T  I  K  L  A  M  K  S  Y  N  T  K  K  Y  Y  D  A  N

421  TATACAAATAAGTGCAATTATAGCTACCGCTACGACATGTGAAGATGGATTTAAGGAGAA
131   I  Q  I  S  A  I  I  A  T  A  T  T  C  E  D  G  F  K  E  K

481  AGAAGGTGCTGTGTCACCGTTAACTATGAGAAATGATAATACTTTTCAATTATCTGCAAT
151   E  G  A  V  S  P  L  T  M  R  N  D  N  T  F  Q  L  S  A  I

541  TGCACTTTCTGTTATGAATCTTGTTATGAATAATAATGGATAAATTGGTCGATATTGATC
171   A  L  S  V  M  N  L  V  M  N  N  N  G

601  AAGTACTTGTTCGTGCATTAAAAAAAAAAAAAAAAAAA
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Figure 4 Nucleotide sequence of the sts15 cDNA and its deduced amino acid sequence
The start and stop codons are shown in boldface and putative N-glycosylation sites are underlined (Accession number X80472).

gene, or a homologue of *sts15*, is present as one or two gene copies in these plant species. With the sts15 probe, two very strong hybridising EcoRI fragments were detected and four strong HindIII fragments were visible in digests of *S. tuberosum* genomic DNA (Figure 5B). Sequence analysis had revealed the presence of an internal HindIII restriction site in the sts15 cDNA sequence. The number of DNA fragments hybridising to the sts15 probe indicate that the *sts15* gene belongs to a small gene family in dihaploid *S. tuberosum*.

To determine the conservation of expression of the *sts15* gene in these plant species, RNA from pistils was isolated and analysed by Northern blot hybridisation. Sts15 transcripts were detectable in *S. tuberosum* and at a low level in pistils of *N. tabacum* (Figure 5C). In contrast

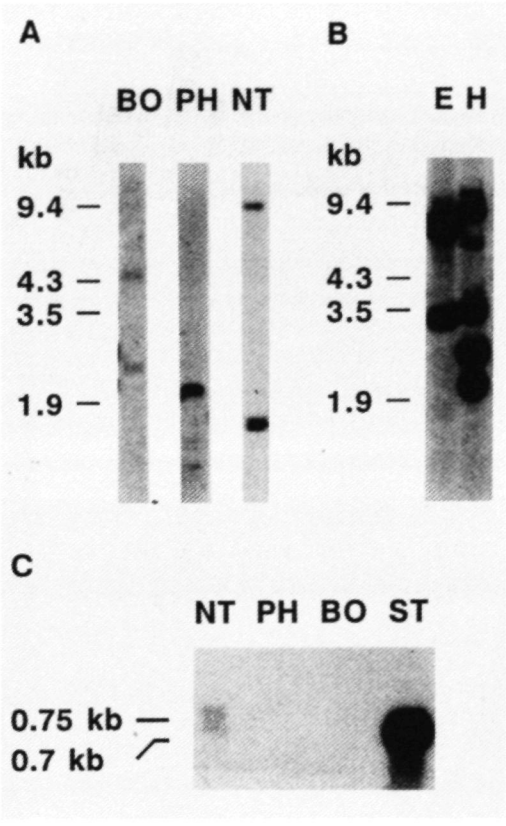

Figure 5 Conservation of the *sts15* gene in other plant species

A. Southern blot analysis of genomic DNA of the following species: BO, *Brassica oleracea*; PH, *Petunia hybrida*; NT, *Nicotiana tabacum*. Genomic DNA was digested with EcoRI and probed with sts15 cDNA. Molecular size of digested lambda DNA fragments are indicated at the left. Following hybridisation the blot was washed in 1x SSC, 0.1% SDS at 60°C and used for autoradiography. B. Southern blot analysis of genomic DNA of *Solanum tuberosum*. Genomic DNA was digested with EcoRI (E) or HindIII (H) and probed with sts15 cDNA. Molecular size of digested lambda DNA fragments are indicated at the left. Following hybridisation the blot was washed in 1x SSC, 0.1% SDS at 60°C and used for autoradiography. C. Northern blot analysis of the conservation of the *sts15* expression. Total RNA was isolated from pistils of: NT, *Nicotiana tabacum*; PH, *Petunia hybrida*; BO, *Brassica oleracea*; ST, *Solanum tuberosum*. The size of the hybridising transcripts is indicated at the left. Following hybridisation the blot was washed in 1x SSC, 0.1% SDS at 55°C and used for autoradiography.

to potato, sts15 cDNA hybridised with two transcripts of 0.7 kb and 0.75 kb in tobacco. Shorter exposure of the blot revealed that the very strong hybridisation signal observed in *S. tuberosum* pistils consists of transcripts of one length.

Discussion

For a better understanding of the process of pollination, it is important to identify genes specifically expressed in all different tissues of the pistil and establish their functional role during pollination. All the pistil-specific genes previously isolated are expressed in the stigmatic tissue and/or in the transmitting tissue of the pistil (Gasser and Robinson-Beers, 1993; Nasrallah and Nasrallah, 1993; Sims, 1993).

As it can be concluded from the Northern blot analysis, *sts15* is a gene highly expressed in pistil. Since *sts15* homology at the genomic level was shown in *B. oleracea*, *P. hybrida*, and *N. tabacum*, *sts15* is a member of a small gene family which is conserved during evolution. Conservation at the gene expression level differs for the tested species; only potato and tobacco showed expression of the *sts15* gene or a gene homologous to *sts15*. Despite the high level of conservation at the genomic level, the expression of *sts15* is not conserved in the *Solanaceae*. In contrast to the presence of a transcript of 0.7 kb in potato, an additional transcript of 0.75 kb is present in tobacco pistils (Figure 5C). This may have been caused by the use of two different polyadenylation sites in the tobacco gene.

The *sts15* gene is the first pistil-specific gene described that is expressed in the cortex of the pistil. The localisation of *sts15* expression in the stylar cortex (Figure 3) is, so far, unique among pistil-specific genes. However, localisation of expression of *sts15* in the pistil epidermis as well cannot be excluded. The other described genes with expression in the cortex are the flower-specific *fst* gene isolated by Gu *et al.* (1992) and the *ta20* gene described by Koltunow *et al.* (1990). The *fst* and *ta20* genes are expressed in the cortex of the style, but also in petals, stamens, and other parts of the pistil. Furthermore, in some species the cortex is demarcated from the transmitting tissue by a zone of callose-containing cells around the stylar channel (Knox, 1984). In this zone, Wu *et al.* (1993) showed the accumulation of celp (cys-rich extensin-like protein) mRNAs.

The localisation of *sts15* expression primarily in the stylar cortex combined with its maximum expression just before anthesis suggests that the gene products of *sts15* do not directly play a role in processes during or after pollination even though the temporal expression pattern is similar to that of the potato S-genes (Kaufmann *et al.*, 1991). The deduced protein

sequence of the sts15 cDNA has no homology with other sequences in the databases and could not, therefore, elucidate a possible function for *sts15*. The specific expression of *sts15* in the parenchymatous tissue of the cortex suggests that these celltypes are highly differentiated and may play a different role than in other organs.

Most of the pistil-specific genes isolated so far are expressed in the tissues that come in close contact with the pollen or pollen tube (stigma and transmitting tissue). However, the mature form and proper functioning of the stigma and style depend on a developmental programme that includes all tissues of the pistil. The spatial and temporal patterns of expression of *sts15* strongly suggest that the gene is developmentally regulated and may have a specific function in the cortex. To further understand the process of pollination, knowledge of both the tissues in direct contact with the pollen tubes is necessary. To this end, greater effort should be made to isolate and characterise genes involved in the development of the pistil cortex and epidermis.

Acknowledgement

We thank Wim van den Brink and Gerard van der Weerden for excellent greenhouse work and Theo Laeijendecker for photography. Critical manuscript editing was provided by Anton Croes and Celestina Mariani. This work was supported by the EC Bridge programme BIOT 900172.

Materials and Methods

Plant material

Plants of self-incompatible dihaploid ($2n=2x=24$) *Solanum tuberosum* clones heterozygous with respect to the S-alleles (S_1S_3 and S_2S_4) were kindly supplied by Professor E. Jacobsen (Wageningen Agricultural University, The Netherlands). Cuttings were grown in climate

chambers at 20°C under a light/dark regime of 16/8 h

Pistils (without the ovary) were collected from flowers at anthesis Mature pollen was collected at anthesis and dried at room temperature for 48 h in open petri-dishes (Van Herpen, 1984) Pollinations were carried out just before anthesis and pistils were collected after 24 hours All tissues were frozen in liquid nitrogen and stored at -80°C

Development of potato flowers was divided into four stages according to Kaufmann *et al* (1991) Stage 1 is characterised by a closed green bud (3-4 mm), stage 2 is a flower bud (5-6 mm) still closed in which the anthers are turning yellow Stage 3 (8-9 mm) is that of the partly opened flower bud in which the petals and anthers are coloured Stage 4 corresponds to flowers at anthesis (over 10 mm).

RNA and DNA extraction

Total RNA was isolated using the method of Frankis and Mascarenhas (1980) with slight modifications according to Goldberg *et al* (1981) Plant tissue frozen in liquid nitrogen was homogenised in 4 ml RNA extraction buffer (100 mM TRIS-HCl pH 8 0, 50 mM EDTA, 1% SDS, 0 1 mM NaCl, 50 mM β-mercaptoethanol and 1% Tri-iso-propylnaphtalene sodium salt (Kodak)) and an equal volume of phenol, and incubated at 60°C for 10 minutes Additional phenol/chloroform/isoamylalcohol (25 24 1) extractions were performed until no interface was present anymore RNA was obtained with successive ethanol and 2 M LiCl precipitations The same procedure was used to extract high molecular weight DNA from young leaves except that β-mercaptoethanol was omitted

cDNA library construction and differential screening

Cross-pollinated ($S_1S_3xS_2S_4$) pistils were harvested 24 h after pollination Poly(A)$^+$ RNA was isolated using a PolyATtract poly(A)$^+$ mRNA isolation kit (Promega) cDNAs were synthesised from poly(A)$^+$ RNA, using a Uni-ZAP XR cDNA synthesis kit (Stratagene), according to the protocols of the manufacturer The library was packaged using Gigapack II gold packaging extracts (Stratagene) The cDNA library was differentially screened on nitrocellulose with ^{32}P-labelled single-stranded cDNA probes The cDNA probes were prepared from either cross-pollinated pistil poly(A)$^+$ RNA and unpollinated pistil poly(A) $^+$

RNA (Sambrook *et al.*, 1989) The ExAssist/SOLR *in vivo* excision system of Stratagene was used for automatic excision of the positive cDNA clones from lambda ZAP II to yield the pBluescript II SK(-) vector in XL-1 Blue *E. coli* cells (Stratagene).

The isolated sts15 cDNA clone was not full-sized Therefore, the 5'-Amplifinder RACE kit from Clontech was used to isolate the 5' end of the sts15 cDNA clone. Two nested internal primers were designed (VE5, 5'-TCGAAATCATTCCAAGGCCACGGAG-3', VE6, 5'-GCACATTGACTCGCGGAGCAGCTTG-3'). The primer VE5 was used to synthesise cDNA and the primer VE6 was used for PCR amplification of the 5' cDNA end according to the protocols of the manufacturer. The PCR fragments were cloned with the TA Cloning System from Invitrogen into the pCRII vector.

DNA manipulations and sequence analysis

Plasmid DNA was prepared as outlined in Sambrook *et al.* (1989) Radioactively labelled probes were prepared from cDNA fragments in low melting point agarose using the random-primer labelling system (Church and Gilbert, 1984). Nucleotide sequence analysis was carried out by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the T7 DNA polymerase sequencing system of Pharmacia Both nucleotide and deduced protein sequences were analysed using the University of Wisconsin Computer Group programmes (Devereux *et al.*, 1984).

Northern blot analysis

Equal amounts of total RNA (10 µg) from different tissues were electrophoretically separated on 1 5% agarose gels and transferred to Hybond-N (Amersham) according to Sambrook *et al.* (1989) The sts15 cDNA was used as a probe Hybridisation was performed for 20 hours at 55°C in 6x SETS (1xSETS is 0.15 M NaCl, 0.02 M TRIS-HCl pH 7 8, 1 mM EDTA), 5x Denhardt's (1x Denhardt's is 0 02% Ficoll, 0 02% PVP and 0.02% BSA), 0 1% SDS and 75 µg/ml denatured herring sperm DNA Washing was carried out at 55°C in 1x SSC, 0 1% SDS or at 55°C in 4 x SSC, 0.1% SDS. The filters were exposed to Valca HPX44 X-ray films with an intensifying screen at -80°C.

Southern hybridisation of genomic DNA

Genomic DNA (30 µg) was digested with EcoRI or HindIII and electrophoretically separated on 0 7% agarose gels Southern blotting and hybridisation were performed on Hybond-N as described by Amersham. The sts15 cDNA was used as a probe.

In situ hybridisation

In situ hybridisation was performed according to Reijnen *et al.* (1991). Pistils at stage 3 were collected and fixed in GPMP buffer (2% glutaraldehyde, 2% paraformaldehyde, 50 mM mannitol, 18 5 mM K2HPO4 and 10 5 mM KH2PO4, pH 7.0) After dehydration, the pistils were embedded in paraffin (Paraclean, Klinipath), cut in 10 µm thick sections, and mounted on slides (Angerer *et al*, 1987). The mounted slides were deparaffinised, hydrated and (pre)hybridised according to Cox *et al.* (1984) and Hanson *et al.* (1989) with slight modifications (Reijnen *et al.*, 1991) The probes used in the *in situ* hybridisation were ^{3}H-labelled antisense and sense RNA, with an activity of $2x10^{6}$ cpm. The probes were synthesised by *in vitro* transcription of sts15 cDNA cloned in the pBluescript II SK(-) vector. The samples were immersed in a light-sensitive emulsion (Ilford L4) After an exposure of 21 days at 4°C the developed silver grains were detected in the emulsion by measuring the reflectance with the BioRad MRC-600 Confocal Laser Scanning Microscope.

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CHAPTER 3

Molecular analysis of a pistil-specific gene expressed in the stigma and stylar cortex of *Solanum tuberosum*

published in *Plant Molecular Biology* (1996) 30: 171-176

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Abstract

A gene, *sts14*, encoding a highly expressed mRNA in pistils of *Solanum tuberosum*, was isolated. Northern blot and *in situ* analyses demonstrated that the gene was expressed throughout pistil development in both the stylar cortex and the stigma. The deduced STS14 protein displays similarity to the pathogenesis-related PR-1 proteins. A possible function for protection or guidance of the pollen tubes through the pistil is discussed.

Introduction

Pollination and subsequent fertilisation in flowering plants comprise the following events the pollen lands on the stigma, germinates and the pollen tube grows through the style towards the ovary in which fertilisation takes place During this sequence of processes the pistil plays an essential role in the guidance, nourishment and protection of the pollen tubes Our goal is to elucidate this role at a molecular level

The pistil delivers compounds like myo-inositol (Kroh *et al*, 1970), flavonols (Mo *et al*, 1992, Ylstra *et al*, 1994), arabinogalactan-proteins (Cheung, 1995, Labarca and Loewus, 1973) to the pollen, sustaining germination on the stigma and growth of the pollen tubes through the style Moreover, the pistil is thought to deliver compounds for the guidance of the pollen tubes through the stigma and transmitting tissue (Cheung, 1995, Herrero and Arbelo, 1989, Hulskamp *et al*, 1995, Lord and Sanders, 1992) and compounds that prevent pollen tube growth in the stylar cortex Due to the physical penetration of non-aseptic pollen tubes, the pistil is wounded and, as a defence mechanism, should be able to produce, before pollination, compounds toxic for pathogens, such as proteinase inhibitors (Atkinson *et al*, 1993), defense-related thionins (Gu *et al*, 1992, Karunanandaa *et al*, 1994, chapter 4) and pathogenesis-related proteins (Constabel and Brisson, 1995, Leung, 1992, Lotan *et al*, 1989, Neale *et al*, 1990, Ori *et al*, 1990)

We have set out to study the molecular events involved in post-pollination processes in the

pistil which are essential for pollen tube growth with respect to guidance, nourishment and protection.

Results

A cDNA library of *Solanum tuberosum* pollinated pistils was differentially screened as described previously (chapter 2, Van Eldik *et al.*, 1995) to isolate cDNA clones representing genes that are highly expressed in the pollinated pistil. The characterisation of one of these clones, sts14, is described in this chapter.

Sts14 represents a 0.75 kb mRNA transcript in potato pistils (pistil refers to stigma together with style) (Figure 1A). The expression level of the gene did not change after pollination. The presence of sts14 or homologous transcripts was also detected in pistils of tobacco and *Petunia* (Figure 1B). Analysis of the temporal expression of the *sts14* gene indicated that transcripts accumulate in pistils of 3-4 mm flower buds (120 h before anthesis) and were more abundant towards the end of flower development, with a maximum at anthesis (Figure 1C).

The spatial distribution of sts14 mRNA within the pistil was shown by *in situ* hybridisation (Figure 2). An sts14 antisense RNA probe was hybridised *in situ* with longitudinal sections of the mature pistil and the hybridisation signal was analysed by confocal laser scanning microscopy (CLSM). An overview of a pistil with bright-field illumination is given in Figure 2A. The sts14 antisense RNA probe hybridised with mRNA in the stigmatic tissue and the papillar cells (Figure 2B). A hybridisation signal was also detected in the stylar cortex but not in the transmitting tissue (Figure 2C). In none of the experiments, the sts14 sense RNA probe gave a signal above the background (data not shown). The results show that sts14 mRNA is localised in the stigma and in the cortex, the same tissues in which another potato pistil mRNA (sts15) accumulates (chapter 2, Van Eldik *et al.*, 1995).

Southern blot analysis revealed that *sts14* is a single copy gene (data not shown). For the determination of the complete open reading frame of sts14 a λEMBL 3a genomic library of *S. tuberosum* cv. Datura (a gift of Dr. J. Brunstedt, MARIBO seeds, Denmark) was screened

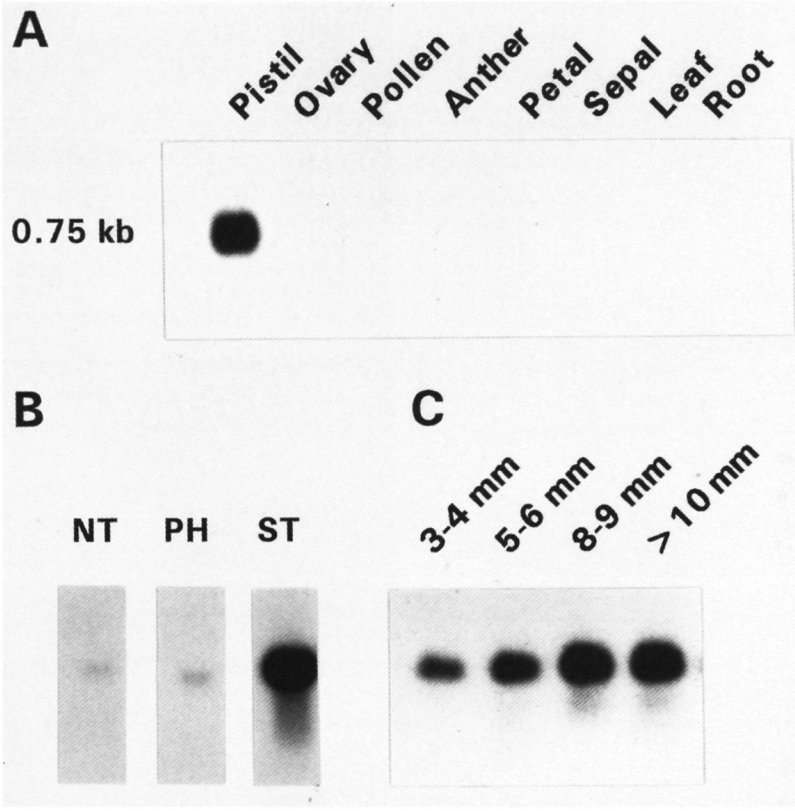

Figure 1 Northern blot analysis of *sts14* gene expression

A. Expression of the *sts14* gene in different potato organs. B. Expression of the *sts14* gene in pistils of solanaceous plant species. C. Expression of the *sts14* gene during pistil development. Flower buds at different developmental stages (chapter 2, Van Eldik *et al.*, 1995) were used. Total RNA (10 µg per lane) was isolated, fractionated by agarose/formaldehyde gel electrophoresis and transferred to nylon membranes. Following hybridisation to the sts14 cDNA probe, the blots were washed in 0.5x SSC, 0.1% SDS at 60°C and used for autoradiography. The size (kb) of the hybridising band is indicated. NT, *Nicotiana tabacum*; PH, *Petunia hybrida*; ST, *Solanum tuberosum*.

using the sts14 cDNA clone as a probe. A comparison of the sequence of the sts14 cDNA with that of the open reading frame of the genomic clone (Figure 3) indicated a homology of 96%. This slight variation in coding sequence may be attributed to differences between the plant

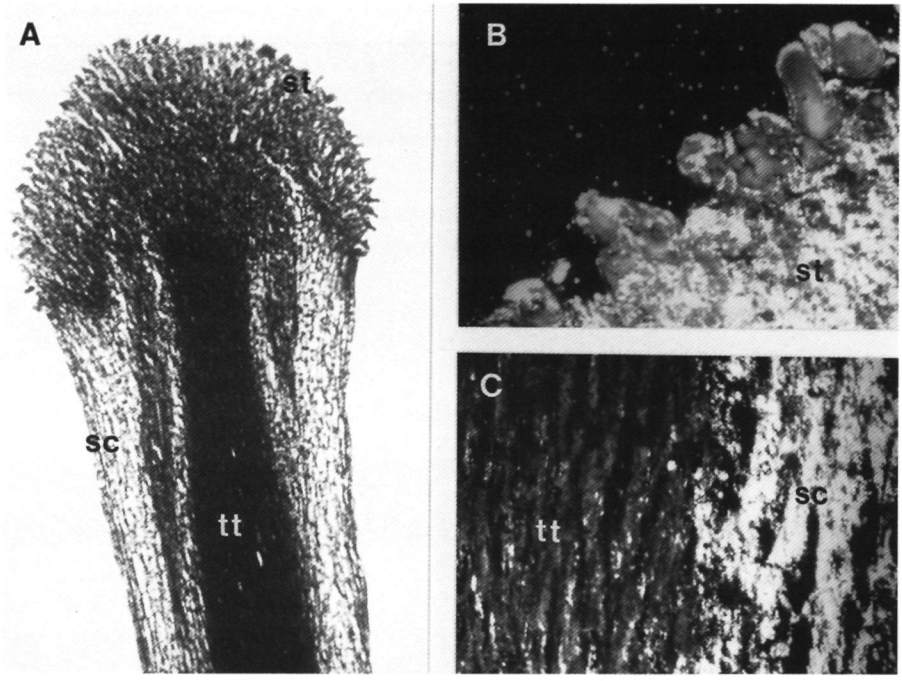

Figure 2 Spatial expression of the *sts14* gene in mature potato pistils

A. Bright-field illumination of a longitudinal section of a potato pistil stained with toluidine blue. B-C. *In situ* hybridisation of sts14 in longitudinal 10 µm sections stained with ethidium bromide (chapter 2, Van Eldik *et al.*, 1995). B. Antisense sts14 probe hybridising with the stigma of the pistil. C. Antisense sts14 probe only hybridising within the cortex, no hybridisation signal in the transmitting tissue. Magnification: A, 20x; B-C, 520x. Stylar cortex (sc), transmitting tissue (tt), stigma (st).

varieties used for cDNA library (a self-incompatible dihaploid potato clone) and genomic library preparation. No introns were present in the *sts14* open reading frame, resulting in one continuous coding region of 642 nucleotides. This sequence together with the 107 nt 3' untranslated region of the cDNA clone, corresponds to the mRNA length on Northern blot (Figure 1A). An in frame stop codon just before the ATG at position 560 of the nucleotide sequence (Figure 3) indicates that this ATG is probably the starting methionine. The *sts14* gene encodes a protein of 214 amino acids with a predicted molecular weight of 24 kDa and an

isoelectric point of 8 6 A potential secretory signal cleavage site (Von Heijne, 1986) is located between amino acids 19 and 20. The protein sequence contains two characteristic domains, a tyrosine-isoleucine repeat (YIYIYIY) at position 13-19 and a stretch of 7 proline residues (position 59-65). In the 3' untranslated region a polyadenylation signal sequence (Joshi, 1987) is located 56 bp downstream of the stop codon (TGA)

Homology searches in computer databases revealed that the STS14 protein shows the highest homology with PR-1 proteins In Figure 4, the predicted STS14 protein sequence is presented and compared with the six most closely related amino acid sequences of PR-1 proteins of tobacco, *Arabidopsis*, barley, maize, tomato and *Medicago truncatula* with amino acid identities ranging from 38% to 43%. The identity of PR-1 proteins between other species ranged from 49% between barley and tobacco, to 92% among the different tobacco PR-1 proteins. As it may be concluded from the data presented in Figure 4, the region with highest PR-1 homology is located at the C-terminal part of the STS14 protein sequence. This is also the part of the protein where 5 out of 7 cysteine residues perfectly match in the aligned PR-1 sequences.

A tobacco PR-1 antibody showed the induction of PR-1 protein synthesis after treatment of tobacco styles with a pathogenesis elicitor (Lotan *et al.*, 1989). Therefore, we treated open flowers of potato by spraying with salicylic acid (5 mM), but we did not detect an overexpression of the *sts14* gene in pistils or whole flower buds after the treatment (data not shown) This indicated that there is no similarity between the *sts14* gene and PR-1 genes with regard to salicylic acid inducibility in pistils. However, a regulation at the translational level may not be excluded

Discussion

The fact that STS14 has homology with PR-1 proteins suggests that it may have a function to protect the outer tissues of the pistil from pathogen attack Also an other PR-like gene (PR-10a) is expressed in the stigma of potato (Constabel and Brisson, 1995) and a PR-2 protein

```
   1 CTATAATTGGATCATATCTGTAACTTTTAACATGTGAAGCTATTTAGTGTAAAAATTAAA
  61 TTGGATGCTGGCTATAATATTGTGTTGTTGGTGTGTTGTACATGAAGAAATTCCTCTGGC
 121 TTGAAGTTGATAGAAAATTAGGAACTCCTATCATTGCCATGCTCTCCATTTTTTTTTCTT
 181 CATGATTTTGTTGGTTTGGCGGGCTGCTTCTTACAAGTGAAATAAATATTTCTCAGGTTT
 241 TACTATATCAATGTCAGTGCAAAGACCATATCTTCAAGAGTTCACATCAAAGATTTCAGA
 301 CATAAATCATAAATTATATATATGCTGGTAGATTGGCAATTGAATGTTCCTTGCATCTTT
 361 CTGTTTTATTTATTGATTGATTATTAAATTTAGCAAGCAATTATAATAGTAATCATGTAA
 421 GAACTTCAACGGTTGTTTATATTAACATGCTTGTGAGTCTTCATGGTGGAATGATGAAGC
 481 GAAACCAGAAATGTATCCCTATTTATCAAAGTCTTGGAAGATGATTTTCATGCAAGTTTT
 541 TCTGTTTTAGGTGAATAACATGTTCGTGCTAAGCACGGCCATGGCCTGTCTAGTATATAT
   1                     M  F  V  L  S  T  A  M  A  C  L  V  Y  I

 601 ATATATATATATATATGATGAAGAAAAGAAAAGAGAGTTGAAAGTAAGAAACAAAATGAC
  15  Y  I  Y  I  Y  D  E  E  K  K  R  E  L  K  V  R  N  K  M  T

 661 CAACTTATTGTTCTTCCAATTCCTCCTATTAACCACTGCTTCATCCTTAACTCACATCTC
  35  N  L  L  F  F  Q  F  L  L  L  T  T  A  S  S  L  T  H  I  S

 721 TGCACAGACAGTTCCACCACCACCACCACCGCCGACATCCGCTGCAACTCCACCGTCCCG
  55  A  Q  T  V  P  P  P  P  P  P  P  T  S  A  A  T  P  P  S  R

 781 CGCGGCGCAAGAATTCTTGGATGCACATAACAAGGCAAGAAGTGAAGTGGGTGTTGGCCC
  75  A  A  Q  E  F  L  D  A  H  N  K  A  R  S  E  V  G  V  G  P

 841 ATTGACATGGAGTCCAATGTTAGCAAAAGAAACTAGCCTTCTTGTTCGTTACCAAAGGGA
  95  L  T  W  S  P  M  L  A  K  E  T  S  L  L  V  R  Y  Q  R  D

 901 CAAACAAAATTGCAGCTTTGCTAATTTAAGTAATGGCAAATATGGTGGCAATCAATTATG
 115  K  Q  N  C  S  F  A  N  L  S  N  G  K  Y  G  G  N  Q  L  W

 961 GGCTAGTGGTACGGTGGTGACCCCACGAATGGCTGTCGATTCTTGGGTTGCTGAGAAGAA
 135  A  S  G  T  V  V  T  P  R  M  A  V  D  S  W  V  A  E  K  K

1021 ATTTTATAACTATGAAAATAATTCATGTACAGGGGATGATAAGTGTGGAGTTTATACCCA
 155  F  Y  N  Y  E  N  N  S  C  T  G  D  D  K  C  G  V  Y  T  Q

1081 AATTGTTTGGAAGAAATCAATAGAATTGGGTTGTGCGCAACGTACTTGTTACGAAGGACC
 175  I  V  W  K  K  S  I  E  L  G  C  A  Q  R  T  C  Y  E  G  P

1141 TGCTACTCTTACTGTATGTTTCTATAATCCACCTGGAAATGTAATTGGAGAGAAACCTTA
 195  A  T  L  T  V  C  F  Y  N  P  P  G  N  V  I  G  E  K  P  Y

1201 TTGATCTGTGATTTCACATGATTTGTAAGGTAACTTTTGTGTTTTCAAGTTTGTAGCTTA
1261 TAATAAGAAGTTACTCTGTTCATTTGCTGCTATTCTCCATTTACTTTTTCACTTTCAGTA
1321 TAATTAGTGCAGGACTGCAG                                    ▲
```

Figure 3 Sequence and deduced protein of the *sts14* gene

DNA sequence of the potato *sts14* gene and the predicted amino acid sequence (Accession number X82652) The putative start codon and stop codon are shown in boldface In the downstream, untranslated region, the potential polyadenylation signal is underlined, and the site of polyadenylation of the cDNA clone is shown by an arrow head The first underlined 19 amino acids represent the predicted secretory signal sequence (Von Heijne, 1986)

```
                1                                                    50
S.tuberosum STS14  MFVLSTAMACLVYIYIYIYDEEKKRELKVRNKMTNLLFFQFLLLTTASSL
N.tabacum PR-1C    ..............................MSS--LVST-LLFLII
A.thaliana PR-1    ............................MNF-GYSR-LIVFVALVGAL
H.vulgare RB-1     ............................MQTPK-AILLA.-AMA-A.M
Z.mays PRMS        ...........................MEASNK-AVLLLW-VMA-ATA
L.esculentum P4    ...........................MGLF-ISLLLTC-. MVLAI
M.truncatula PR-1  ...........................MSFRCFS-AL----L-FI-H

                51                                                   100
S.tuberosum STS14  THISAQTVPPPPPPPTSAATPPSRAAQEFLDAHNKARSEVGVGPLTWSPM
N.tabacum PR-1C    S-SCH........AQN-.........Q-DY-----T--AD---E----DDQ
A.thaliana PR-1    VLP-K........AQD-.........P-DY-RV--Q--GA-----MQ-DER
H.vulgare RB-1     VNL-Q........AQN-.........P-DYVSP--A---A----AVS--TK
Z.mays PRMS        V-P-Y........SEN-.........P-DY-TPQ-S--AA-----V---TK
L.esculentum P4    F-SCE........AQN-.........P-DY-AV--D--AQ-----MS-DAN
M.truncatula PR-1  VSA-Y........I-NKKSFKSRSFKNQ--IPQ-I--AA--LR--V-DDK
                                                 * **  **     *

                101                                                  150
S.tuberosum STS14  LAKETSLLVRYQRDKQNCSFANLSNGKYGGNQLWASGTVV.TPRMAVDSW
N.tabacum PR-1C    V-...AYAQN-ASQLAADCNLVH-H-Q--E-LA-G--DF.L-AAK--EM-
A.thaliana PR-1    V-...AYARS-AEQLRGNCRLIH-G-P--E-LA-G--D..LSGVS--NM-
H.vulgare RB-1     -Q...AFAQN-ANQRI-DCKLQH-G-P--E-IF-G-AGADWKASD--N--
Z.mays PRMS        -Q...QFAEK-AAQRAGDCRLQH-G-P--E-IF-G-AGFDWKAVD--R--
L.esculentum P4    --...-RAQN-ANSRAGDCNLIH-GA..-E-LAKGG-D..F-G-A--QL-
M.truncatula PR-1  -T...HYAQW-ANQRR-DCALEH---P--E-IF-G--.-GWN-AQ--SA-
                          *              *    * *             **  *

                151                                                  200
S.tuberosum STS14  VAEKKFYNYENNSCTGDDKCGVYTQIVWKKSIELGCAQRTCYEGPATLTV
N.tabacum PR-1C    -N--QY-AHDS-T-AQGQV--H---V--RN-VRV---RVQ-NN- GYIVS
A.thaliana PR-1    -S--AN---AA-T-N-..V--H---V--R--VR----KVR-NN- G-IIS
H.vulgare RB-1     -S---D-D-GS-T-AAGKV--H---V--RA-TSI---RVV-NNNRGVFIT
Z.mays PRMS        -D--QW---AT---AAG-V--H---V--RATTSI---RVV-RDNRGVFII
L.esculentum P4    -S-RPD---AT-Q-V-GKM--H---V--RN-VR---GRAR-NN- WWFIS
M.trucatula PR-1   -D--Q----WH---VDGEM--H---V--GSTTKV---SVV-SDDKG-FMT
                   * *   *    * *     ** *** **      **    *

                201         215
S tuberosum STS14  CFYNPPGNVIGEKPY 214
N.tabacum PR-1C    -N-D-------KS-- 168
A.thaliana PR-1    -N-D-R--YVN---- 161
H.vulgare RB-1     -N-E-R--I--Q--- 164
Z.mays PRMS        -N-E-R--IA-M--- 167
L.esculentum P4    -N-D-V--WV--R-- 159
M.truncatula PR-1  -N-D----YY--R-- 173
                   * * * **     **
```

Figure 4 Alignment of STS14

Alignment of the deduced amino acid sequence of *sts14* with that of PR-1 proteins of *Nicotiana tabacum* (Pfitzner and Goodman, 1987), *Arabidopsis thaliana* (Uknes *et al* , 1992), *Hordeum vulgare* (Muradov *et al* , 1993), *Zea mays* (Casacuberta *et al.*, 1991), *Lycopersicon esculentum* (Van Kan *et al* , 1992) and *Medicago truncatula* (Szybiak-Strozycka *et al.*, 1995). Sequences were aligned using the PILEUP programme of the University of Wisconsin Computer Group (Devereux *et al.*, 1984) Sequence positions are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence Amino acids identical to those in STS14 are indicated by dashes Dots represent a gap Residues that are identical between STS14 and all PR-1 proteins are marked by an asterisk

was localised in the transmitting tissue of tobacco pistils (Ori *et al*, 1990) However, an indirect role of STS14 in the cortex in controlling the guidance of pollen tubes cannot be excluded, as pollen tubes of *Arabidopsis* can grow within the cortical tissue at very early stages of pistil development (Kandasamy *et al*, 1994) This suggests that during pistil development gene products may be synthesised in the cortex and prevent pollen tubes to grow within the parenchymatic tissue of the stylar cortex The homology to defence-related PR-1 proteins, combined with the temporal and spatial expression patterns, suggests that STS14 may be involved in processes of this kind However, the presence of STS14 in the stigma is not in complete conformity with this possible function Further research is necessary to define the function of the *sts14* gene product in the pistil and, in general, to proceed with the characterisation of genes and their products expressed in pistil tissues, which can contribute to the understanding of how these tissues function during pistil development and fertilisation

Acknowledgements

We wish to thank Wim Vriezen for his excellent technical assistance, Wim van den Brink for culturing the plants, and Dr A F Croes and Professor C Mariani for critically reading the manuscript This work was supported by the EC Bridge programme BIOT 900172

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CHAPTER 4

Pistil-expressed genes in *Solanum tuberosum*: isolation, characterisation and tissue-specific expression

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Abstract

The reproductive function of the pistil requires the production of compounds essential for pollen tube growth. For a better understanding of the role of the pistil during pollen tube growth, we have set out to analyse the genes expressed in pollinated pistils by cold-plaque screening of a pollinated pistil cDNA library. This resulted in the isolation of six cDNAs predominantly expressed in the pistil. The characterisation and tissue-specific expression of four cDNAs is described in this chapter. The pistil-specific cp37 cDNA contained no open reading frame and showed no significant similarity with any previously reported sequence. Cp67 encoded an alcohol dehydrogenase (ADH)-like protein with all the sequence characteristics of ADHs, although no correlation between cp67 transcript accumulation and ADH activity was present. However, ADH activity and the expression of a previously cloned *sth-11* gene encoding an ADH were both induced by pollination. Cp34 transcripts accumulated specifically in pistils and showed high homology to γ-thionin-like sequences which are thought to be involved in plant defence. Another clone, designated cp71, which encoded a protein similar in sequence to hormone-induced proteins, was expressed in the complete carpel and at lower level in petals. Together, these observations provide new insight into the compounds present in the pistil and the possible functions of the pistil during reproductive development.

Introduction

In flowering plants the pistil is the female reproductive organ. Pollination starts with the deposition of the pollen on the stigmatic surface of the pistil, followed by pollen germination and subsequent pollen tube growth through the stylar part of the pistil towards the ovary. After the pollen tube has entered the ovary, fertilisation occurs in the ovules. During this sequence of processes the pistil plays an essential role in producing compounds necessary for recognition, guidance, protection and nourishment of the pollen tubes. Determination of the nature of genes

ultimately responsible for the formation of these products and the mechanisms controlling their expression greatly increases our understanding of the functions of the pistil tissues In the last few years many pistil-expressed genes have been isolated and characterised, and the encoded proteins are related to the various functions of the pistil The most extensively studied genes encode self-incompatibility-associated glycoproteins which mediate the ability of plants to discriminate between self and non-self pollen (Nasrallah and Nasrallah, 1993, Newbigin *et al* , 1993) Gene products involved in guidance and nourishment include arabinogalactan proteins (Du *et al* , 1996, Lind *et al* , 1996) and extensin-like proteins (Chen *et al* , 1992, Cheung *et al* , 1995, Goldman *et al* , 1992, Wu *et al* , 1995) Gene products in the pistil that could have a function in protection are thionin-like proteins (Gu *et al* , 1992, Karunanandaa *et al* , 1994, Milligan and Gasser, 1995), proteinase inhibitors (Atkinson *et al* , 1993) and pathogenesis-related proteins (Constable and Brisson, 1995, Leung, 1992, Lotan *et al* , 1989, Ori *et al* , 1990, Sessa and Fluhr, 1995, chapter 3, Van Eldik *et al* , 1996) Many of these defence-related gene products are constitutively present and, therefore, could also have other functions in the pistil

The aim of our research was to isolate and characterise genes expressed in pollinated pistils and to establish their functional role during pollen tube growth To achieve these goals, we used a cold-plaque procedure to screen a cDNA library of pollinated pistils of *Solanum tuberosum* and isolated several pistil-specific and pistil-expressed cDNAs The tissue-specific transcript accumulation and sequence analysis of these cDNA clones and the putative functions of the genes are described

Results

To obtain more information regarding genes expressed in pollinated pistils of *Solanum tuberosum*, we initiated a screening of a cDNA library of pollinated pistils of potato according to the cold-plaque screening method as described by Hodge *et al* (1991) For the screening we used a cDNA library made from mRNA of potato pistils 24 hours after pollination, as previously described for the differential screening (chapters 2 and 3) The library was screened

Table 1 Characteristics of pistil-expressed cold-plaque cDNAs

name	cDNA (nt)	mRNA (kb)	expression pattern[b]	homologous gene
cp34	265[a]	0.45	pistil	γ-thionin[d]
cp35	1408	1 4	pistil	flavonol synthase[e]
cp37	650[a]	0 9	pistil	no homology
cp67	1430	1 4	pistil	alcohol dehydrogenase[f]
cp71	279[a]	0 6	pistil	hormone-regulated genes[g]
cp100	1061	1 1	pistil/pollen/leaf pollinated pistils[c]	isoflavone reductase[h]

a not full-length

b as tested on Northern blots

c expression induced after pollination

d 78% amino acid identity with a pistil expressed γ-thionin homologue of *Petunia inflata*

e see chapter 5

f 45% amino acid identity with long chain ADHs

g *gasa/gast1/rsi-1* genes regulated by hormones

h see chapter 6

with single-stranded cDNA of pollinated pistils as a probe, the same mRNA pool as used for preparing the library, and hybridised at low temperature and low stringency conditions In the screening 10 000 plaques were used, whereafter 120 non-hybridising ('cold') plaques were isolated

To identify clones corresponding to transcripts with an expression pattern expected for

```
   1 TTCCAGAATCGATAGTGAGAGCGAAACATTTCTCTTTCTCTCCCCCAACTTGAAGCAAAG
  61 TCCCTCATCTTTACATATTAAGCAAAGTTCAGAAACCTCAATCCGACCTTGTACCATGGA
   1                                                       M  D

 121 CTCAAGCAACCCAAAGGTCATCACATGCAAAGCTGCAGTAGTAGGGAAGGAAGGGGAGAT
   3  S  S  N  P  K  V  I  T  C  K  A  A  V  V  G  K  E  G  E  M

 181 GATAAAGATCGAAGAGATACAAGTGGATCCACCTAAATCAAATGAAGTTCGAATTAAGAT
  23  I  K  I  E  E  I  Q  V  D  P  P  K  S  N  E  V  R  I  K  M

 241 GCTCTTTGCTAGTTTGTGCCACACTGACATTCTCGCTTCCAATGGCTATCCCTATTCTTT
  43  L  F  A  S  L  C  H  T  D  I  L  A  S  N  G  Y  P  Y  S  L

 301 ATTTCCTCGAGTTCTTGGACATGAAGGAGTTGGCATGATAGAAAGTGTGGGAGAAAATGT
  63  F  P  R  V  L  G  H  E  G  V  G  M  I  E  S  V  G  E  N  V
                                                              ↑
 361 GACAAATCTAAAAGAAGGAGATATAGTGATGCCACTTTACTTGGGAGAATGCAAAGAATG
  83  T  N  L  K  E  G  D  I  V  M  P  L  Y  L  G  E  C  K  E  C

 421 CCCAAATTGCAAGTCAGGGAAGTCCAATTTATGTCACAAATATCACTTGACCTTTTCTGG
 103  P  N  C  K  S  G  K  S  N  L  C  H  K  Y  H  L  T  F  S  G

 481 CCTAATGTTGGATGACACATCAAGAATCTCCATTCATGGAGGACAAGTATTATACCATAG
 123  L  M  L  D  D  T  S  R  I  S  I  H  G  G  Q  V  L  Y  H  S

 541 TTTTAGTTGCTCTACTTGGTCGGAATATATAGTTATTAATGCCAACTACGTAATCAAGGT
 143  F  S  C  S  T  W  S  E  Y  I  V  I  N  A  N  Y  V  I  K  V

 601 TGATCCTCAGAAGATTCCGCTTCAACATGCTAGCTTGCTTTGTTGTGGATTTACGACAGG
 163  D  P  Q  K  I  P  L  Q  H  A  S  L  L  C  C  G  F  T  T  G

 661 TTATGGAGCAACATGGAGAGAAGTTCATGTTGAAAAAGGCTCAACTGTTGCTGTACTAGG
 183  Y  G  A  T  W  R  E  V  H  V  E  K  G  S  T  V  A  V  L  G

 721 TCTTGGTGTTGTTGGACTTGGAGCGATTGAGGGAGCTCGAAGTCAAGGGGCATCAAAAAT
 203  L  G  V  V  G  L  G  A  I  E  G  A  R  S  Q  G  A  S  K  I

 781 AATTGGAGTTGATATAAATGAATCGAAACAGGGTAAAGGAGAACTGTTTGGAATGACAGA
 223  I  G  V  D  I  N  E  S  K  Q  G  K  G  E  L  F  G  M  T  D
              ▲     ↑
 841 CTTCATTAACCCTAAAGAATCCATGACATCCGTTTCCGAGATGATAAAAGATGTTACTGA
 243  F  I  N  P  K  E  S  M  T  S  V  S  E  M  I  K  D  V  T  E

 901 AGGCTTAGGGGTTGATTATGTTTTCGAATGTACTGGGATTCCATCCATGCTTAATGAAGC
 263  G  L  G  V  D  Y  V  F  E  C  T  G  I  P  S  M  L  N  E  A

 961 CATTGAAGCCTCAAAATTGGGAATTGGGACAATTGTTGTTATTGGTGCAGGACATGGACT
 283  I  E  A  S  K  L  G  I  G  T  I  V  V  I  G  A  G  H  G  L

1021 TACTAGGGAATTCAACTTAGTTCCCTTATTATGTGGTCGAACATTGAAAGGTTCAATCTA
 303  T  R  E  F  N  L  V  P  L  L  C  G  R  T  L  K  G  S  I  Y

1081 TGGTGGAATAAGACTTCACTCAGATCTTCCTGCCATACTTCATAGATGCGCAACTAAGGA
 323  G  G  I  R  L  H  S  D  L  P  A  I  L  H  R  C  A  T  K  E
```

```
1141 GATTCAACTAAATGAGCTTATAACTCACCAAATTTCGTTGACTGAAATCAACCAATCATT
 343  I  Q  L  N  E  L  I  T  H  Q  I  S  L  T  E  I  N  Q  S  F
                                                      ↑
1201 TGAGTTATTGAAAGACCCCCATTGCGTCAAGATTATTATCAAGTTCTAATAAATGAAACC
 363  E  L  L  K  D  P  H  C  V  K  I  I  I  K  F

1261 AGTTATTAGAACATATTAATGCATAGTGAAATTATTTACTCAATTATGAAATTTGGTTTA
1321 ATTTATGCACCATACATTGTATGCATATGATAAGTAAAATAAACATTGTATTGCATATAT
1381 AAATGTGTATGAAACAAGTGTGTAATAAACATTGCTATTGCAGGATTGAT
```

Figure 1 Nucleotide sequence of the cp67 cDNA and its deduced amino acid sequence

The nucleotide sequence is numbered at the left (Accession number X92179) The sequence positions of the deduced amino acid sequence are relative to the starting methionine and indicated at the left The start and stop codons are shown in boldface In the downstream, untranslated region, the potential polyadenylation signal sequence is underlined The underlined stretch of 15 amino acids represents the zinc-containing ADH signature (Jornvall *et al*, 1987) The NAD binding site is indicated by an arrow head The 22 amino acids conserved in all ADHs are shown in boldface and the three potential N-glycosylation sites are indicated by an arrow

genes related to the various functions of the pistil, the inserts of 33 of these cold-plaques were used as probes on Northern blots prepared with total RNA from pistils, pollinated pistils, pollen and leaves Six different clones hybridised to transcripts only present in pistils or showed a difference in expression level between pollinated and unpollinated pistils (Table 1) The two other 27 cold-plaques did not show a pistil-specific or pollination-related expression pattern, were almost not detectable on Northern blot, or were expressed in all tissues (data not shown) Sequence and expression patterns of four of the six potato cold-plaque (*cp*) genes identified in this screening were analysed The two other cp-clones will be described in more detail in chapters 5 and 6

Cp67 *encodes an alcohol dehydrogenase-like gene predominantly expressed in the pistil*

The cp67 cDNA hybridised to a transcript of approximately 1 4 kb (Table 1) The clone had a length of only 1 1 kb and thus was not a full-length copy of the mRNA Therefore, a 5'-RACE experiment was performed to isolate the 5' end of the cp67 mRNA (see Materials and Methods) The complete sequence of cp67 was found to be 1430 bp in length and contained an open reading frame of 377 amino acids starting from the first translation start codon The

```
                1                                                  50
S.tuberosum CP67   MDSSNPKVITCKAAVVGKEGEMIKIEEIQVDPPKSNEVRIKMLFASLCHT
P.banksiana ADHC2  ......Q--K-----AWAA--PL----VE-A--QAM-----IHYTA----
G.hirsutum ADH2A   .MSTAGQ--R-----AWES-KPLS---VE-A--QKD-----I--T-----
N.tabacum TOBADH1  .RPPAGQ--R-----AWEA-KPLE---VE-A--QKS---L-I--T-----
A.thaliana ADH3    .MATQGQ--------AYEPNKPLV--DV--A--QAG-----I-YTA----
L.sativa LRG5A     MSSTTNQ--R-----AWEA-KPLV---VE-A--QKM-----I--T-----
                          ** *****          **   * **   *** *     ****

                51                                                 100
S.tuberosum CP67   DILASNGY.PYSLFPRVLGHEGVGMIESVGENVTNLKEGDIVMPLYLGEC
P.banksiana ADHC2  -VYFWEAKGQTP----I----AA-VV-----G--D-----T-L-VFT---
G.hirsutum ADH2A   -VYFWDAKGQNP----I----AG-IV-----G--D--P--H-L-IFT---
N.tabacum TOBADH1  -VYFWEAKGQTP----IF---AG-IV-----G--D--P--H-L-VFT---
A.thaliana ADH3    -AYTWS-KD-EG---CI----AA-IV-----G--EVQA--H-I-C-QA--
L.sativa LRG5A     -VYFWEAKGQNPV---I----AG-VV-----G--E-QP--H-L-VFT---
                   *            **   ***  *  ***** **    ** * *    **

                101                                                150
S.tuberosum CP67   KECPNCKSGKSNLCHKYHLTF.SGLM.LDDTSRISIHGGQVLYHSFSCST
P.banksiana ADHC2  G--RH---EE--M-DLLRINTER-V-IS-GKT-F-K.D-KPI--FLGT--
G.hirsutum ADH2A   ----H-L-EE--M-DLLRINTDR-E-IN-GK--F--.N-KPI--FLGT--
N.tabacum TOBADH1  QQ-RH---EE--M-DLLRINTDR-V-IH-GQT-F-K.D-KPI--FVGT--
A.thaliana ADH3    R--KF-----T---G-VRSATGV-I-MN-RK--F-V.N-KPI--FMGT--
L.sativa LRG5A     ---AH---EE--M-DLLRINTDR-V-IH-QK--F--.N-KPIF-FVGT--
                     *  * *   * *          * * *    * *   *    *    **

                151                                                200
S.tuberosum CP67   WSEYIVINANYVIKVDPQKIPLQHASLLCCGFTTGYGATWREVHVEKGST
P.banksiana ADHC2  F---T-AH-GC-A-IN-E.A--DKVCV-S--AS--M--ALNVAKPK----
G.hirsutum ADH2A   F---T-VHVGQ-A-IN-E.A--DKVCV-S--MS--F---VNVAKPK--QS
N.tabacum TOBADH1  F---T-VHSGC-A-I---.A--DKVCV-S--IS--L---LNVAKPT----
A.thaliana ADH3    F-Q-T-VHDVS-A-I--T.A--DKVC--G--VP--L--V-NTAK--P--N
L.sativa LRG5A     F---T-VHVGCLA-IN-L.A--DKVCV-S--IS--L---LNVAKPK---S
                    * * *       *  *   **     * **  ** **         *

                201                                                250
S.tuberosum CP67   VAVLGLGVVGLGAIEGARSQGASKIIGVDINESKQGKGELFGMTDFINPK
P.banksiana ADHC2  ---F---A-----A----IA---R---I-LISERFE-AK---V-E----L
G.hirsutum ADH2A   --IF---A---A-A----VS---R-----L-P-RFELAKN--V-E-V---
N.tabacum TOBADH1  --IF---A---A-A--S-IA---RV--I-L-P-RFNDAKK--V-E-V---
A.thaliana ADH3    --IF---T---AVA---KTA---R---I-IDSK-YETAKK--VNE-V---
L.sativa LRG5A     --IF---A---A-A----IA---R-----L-ANRFELAKK--V-E-V---
                   **  *** ***   **    ***  ** *           **   * **

                251                                                300
S.tuberosum CP67   ESMTSVSEMIKDVTEGLGVDYVFECTGIPSMLNEAIEASKLGIGTIVVIG
P.banksiana ADHC2  DHAKP-Q-V-AEK-D-.----SI----NVKAMIQ-F-SCHD-W-VA-LV-
G.hirsutum ADH2A   DHKKP-Q-V-AEM-G-.---RSV----SIQAMIS-F-CVHD-W-VA-LV-
N.tabacum TOBADH1  DYDKP-QQV-AEM-D-.---RSV----NVNAMIS-F-CVHD-W-VA-LV-
A.thaliana ADH3    DHDKPIQ-V-V-L-D-.----S---I-NV-VMRA-L-CCHK-W--S-IV-
L.sativa LRG5A     DYKKP-Q-V-AEM-N-.---RSV----HIDAMIS-F-CVHD-W-VA-LV-
                         *   * * ***   ** *       * *    * *  *  *
```

```
                  301                                                  350
S tuberosum CP67  AGH GLTREFNLVPLLCGRTLKGSIYGGIRLHSDLPAILHRCATKEIQLN
P banksiana ADHC2 VP-SDAVFQTSPLNF-TE-----TF--NYKPR----GLVEMYLA-K-E-E
G hirsutum ADH2A  VPNKDDAFKTHP-N--NE-----TFF-NYKPRT-I-AVVE-YMN--LE-D
N tabacum TOBADH1 VPNKAMRFKTHPMN--NE-----TFF-NYKPKT---SVVGKYMN -LE-E
A thaliana ADH3   VAAS-QEISTRPFQ-VT--VW--TAF--FKSRTQV-WLVEKYMN---KVD
L sativa LRG5A    VP-KDAVFKTSPLN--NE-----TFF-NYKPR--I-SVVEKYMN--LE-E
                                 *  **   *        *        *

                  351                              384
S tuberosum CP67  ELITHQISLTEINQSFELLKDPHCVKIIIKF    377
P banksiana ADHC2 KF---EV-FAD--KA-DYMLKGESLRC--NLIGN 375
G hirsutum ADH2A  KF---SVPFS---KA--YMLAGEGLRCV-RMVA  379
N tabacum TOBADH1 KF ---VPFS---KA--YMLKGEGLRCM-TMEH  379
A thaliana ADH3   -Y---NL--G---KA-D--HEGT-LRCVLDTSK  379
L sativa LRG5A    KF---ELPFS---KA-D-MLKGEGLRC--RMGE  380
                    ***       **  *
```

Figure 2 Alignment of the deduced amino acid sequence of CP67 with other plant alcohol dehydrogenases

The deduced alcohol dehydrogenase proteins of *Pinus banksiana* (Perry, 1996), *Gossypium hirsutum* (Accession number U49061), *Nicotiana tabacum* (Bucher *et al*, 1995), *Arabidopsis thaliana* (Accession number X82647) and *Lactuca sativa* (Toyomasu *et al*, 1995) are aligned using the PILEUP programme of the University of Wisconsin Computer Group (Devereux *et al*, 1984) Sequence positions are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence Amino acids identical to those in CP67 are indicated by dashes Dots represent gaps Residues that are completely identical among the aligned ADH proteins are marked by an asterisk

predicted molecular mass of this polypeptide was 40 9 kDa (Figure 1) A polyadenylation signal sequence (Joshi, 1987) is located in the 3'-untranslated region 110 bp downstream of the stop codon (TAA)

Sequence database searching revealed that cp67 showed similarity to alcohol dehydrogenases (ADHs) CP67 is most similar to long-chain zinc-containing ADHs (Jornvall *et al*, 1987) and contains the zinc-containing ADH signature G-H-E-x(2)-G-x(5)-G-x(2)-[VAC] between amino acid 68 to 82 (Figure 1) Furthermore, 22 residues conserved among all ADHs (Jornvall *et al*, 1987), were also present (Figure 1) Among these conserved residues are 11 glycines, of which three occur in the coenzyme-binding domain G-x-G-x-x-G (at position 202-207) of dehydrogenases (Wierenga and Hol, 1983) Also the two active-site zinc ligands, Cys-48 and His-69, and Asp-226 that binds to the adenosine ribose of the coenzyme were present

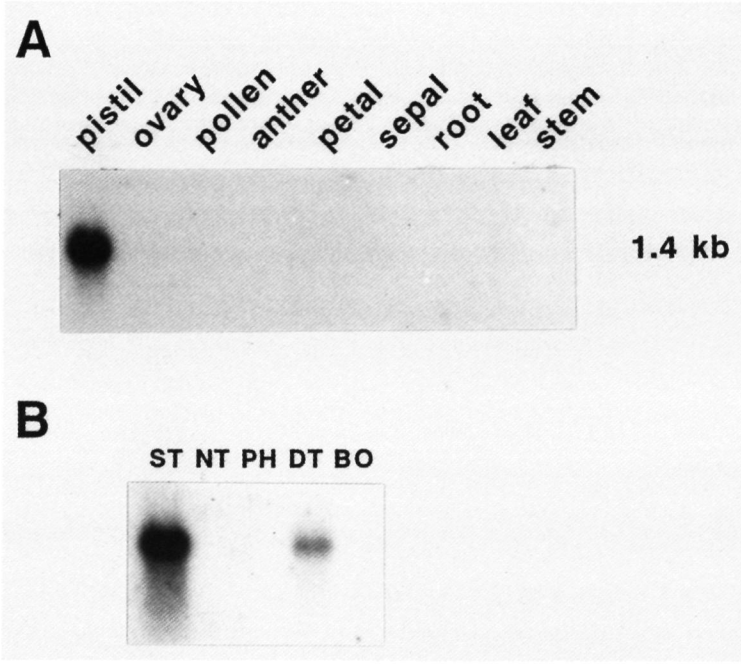

Figure 3 Northern blot analysis of *cp67* gene expression

A. Expression of the *cp67* gene in various potato organs (10 µg RNA per lane). B. Expression of the *cp67* gene in pistils of other plant species (5 µg RNA per lane). Total RNA was isolated, fractionated by agarose/formaldehyde gel electrophoresis and transferred to nylon membranes. After hybridisation to the cp67 cDNA probe, the blots were washed in 0.5x SSC, 0.1% SDS at 55°C and used for autoradiography. The size (kb) of the hybridising transcript is indicated. ST, *Solanum tuberosum*; NT, *Nicotiana tabacum*; PH, *Petunia hybrida*; DT, *Datura*; BO, *Brassica oleracea*.

(Jörnvall *et al.*, 1987). The predicted CP67 protein sequence was compared with five of the most closely related amino acid sequences of plant ADH proteins with amino acid identities ranging from 42% to 45% (Figure 2).

The cp67 *gene is predominantly expressed in pistils*

The initial Northern blot analysis of the *cp67* gene revealed expression in the pistil.

Accumulation of cp67 transcripts did not increase upon pollen tube growth. To further analyse the spatial expression of *cp67*, a Northern blot was prepared with RNA from different flower organs and vegetative parts of potato plants. A single mRNA of approximately 1.4 kb in pistils hybridised strongly to the cp67 probe (Figure 3A). No hybridisation was detected to mRNA from other flower and vegetative tissues. Only after very long exposure a faint signal was seen in all tissues examined. Analysis of the temporal expression of the *cp67* gene in whole flower buds indicated that transcripts started to accumulate in buds of 5-6 mm and were more abundant towards the end of flower development (data not shown).

The conservation of *cp67* gene expression in pistils of other plant species was investigated. As it can be seen in Figure 3B, cp67 transcripts were only detectable in pistils of *S. tuberosum* and *Datura*. No hybridisation of cp67 to mRNA of pistils of *N. tabacum*, *Petunia hybrida* and *Brassica oleracea* was observed. This indicated that the *cp67* gene is not widely spread among other plant species.

ADH activity and transcript accumulation in pistils, before and during pollen tube growth

ADH enzyme activity in the pistil could be expected if cp67 encodes an alcohol dehydrogenase. Therefore, the ADH activity was measured in different potato organs. After native polyacrylamide gel electrophoresis of the soluble proteins and ADH activity staining, ADH activity was only detected in pollen and complete anthers, but not in petals, ovaries and pistils (Figure 4A). When alcohols other than ethanol were used as substrates, there was also no ADH activity in potato pistils. This indicates that there is no direct correlation between the presence of cp67 transcripts and ADH activity.

In *in vitro* germinating pollen of tobacco, ethanol is accumulating. For pollinated pistils it has been suggested that the synthesised ethanol is metabolised by the pistil by the action of ADH (Bucher *et al.*, 1995). To investigate the possibility that in pollinated pistils ADH activity is present, pistils were pollinated and the ADH activity was determined. Anthers and leaves subjected to anoxia, a treatment that induces ADH activity, served as a control. In both pollinated pistils and leaves with oxygen depletion ADH activity was induced (Figure 4B). The ADH activity band present in anoxied leaves migrated faster than the ADH activity band in anthers. In pollinated pistils both bands were detected. This indicates that in leaves anoxia

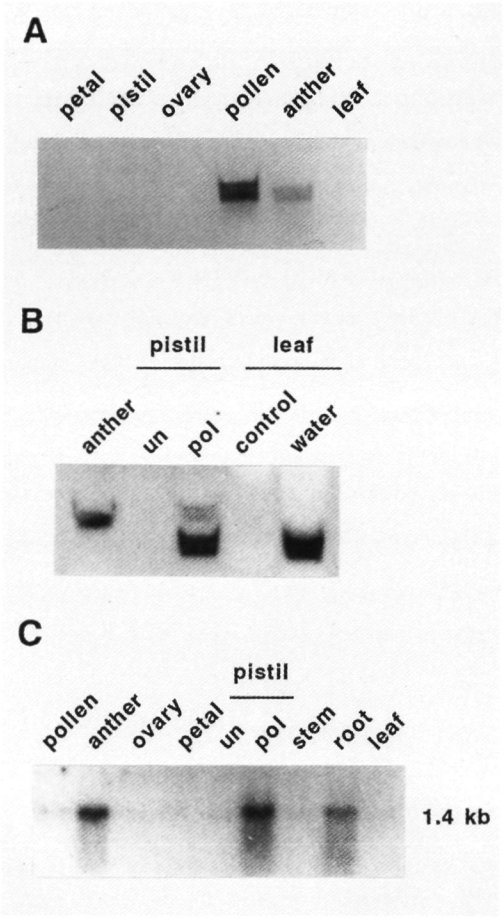

Figure 4 ADH activity and gene expression in potato organs

A. Organ-specific ADH activity. B. ADH activity induced by pollen tube growth. Proteins were isolated and fractionated by native polyacrylamide gel electrophoresis. The gels were stained for ADH activity. C. Northern blot analysis of *sth-11* gene expression in different potato organs. Total RNA (10 µg per lane) was isolated, fractionated by agarose/formaldehyde gel electrophoresis and transferred to nylon membranes. After hybridisation to the sth-11 cDNA probe (Matton *et al.*, 1990), the blots were washed in 1x SSC, 0.1% SDS at 58°C and used for autoradiography. The size (kb) of the hybridising band is indicated. un, unpollinated pistils; pol, pollinated pistils.

induces another ADH isozyme than that present in anthers After pollination, both isozymes are induced in pistils, although the faster migrating form is predominant The induction of ADH enzyme activity by pollen tube growth was not correlated with an increase of *cp67* gene expression, because pollen tube growth did not enhances the expression of this gene (Table 1)

In order to compare the induced ADH activity upon pollen tube growth with gene expression of an *adh* gene, the transcript levels of the ADH encoding potato *sth-11* gene (Matton *et al*, 1990) were analysed on Northern blot Correct enzyme activity of the ADH protein encoded by the sth-11 cDNA was demonstrated *in vitro* (Matton *et al*, 1990) The *sth-11* gene was predominantly expressed in anthers, roots and pollinated pistils, whereas in all other organs tested only a faint hybridisation signal was present (Figure 4C)

The cp34 cDNA encodes a pistil-specific γ-thionin-like protein

Sequence analysis of cp34 cDNA (265 bp) revealed one open reading frame starting at position 3 within the sequence, and resulting in a deduced protein of 51 amino acids (Figure 5A) The cp34 cDNA hybridised to a transcript of approximately 450 nt (Table 1) which indicates that cp34 is probably not a full-length cDNA clone This observation is confirmed by the absence of a starting methionine in the deduced protein sequence and homology analyses (see later) A polyadenylation signal (AATAAA, Joshi, 1987) was not found upstream of the polyadenylation site, however, several AT-rich regions that may perform this function are present in the 3' untranslated region The predicted partial CP34 protein is rich in cysteine (15%) and glycine (14%), but does not contain methionine, tyrosine and tryptophan

A search of the sequence database for sequences similar to cp34 revealed that the CP34 protein shares 78% amino acid identity with a predominantly pistil-expressed γ-thionin-like protein (PPT) from *Petunia inflata* (Karunanandaa *et al*, 1994) Furthermore, amino acid sequence identity was found with other γ-thionin-like sequences (65% to 75%) and at a lower level with two flower-specific γ-thionin-like proteins, both 37%, from tobacco and tomato (Gu *et al*, 1992, Milligan and Gasser, 1995) Remarkable is the perfect conservation of the cysteine amino acids between all sequences Figure 5B shows a comparison of the deduced CP34 protein and the most closely related amino acid sequences of γ-thionin-like proteins of *P inflata* (Karunanandaa *et al*, 1994), *A thaliana*, *Brassica rapa*, *Capsicum annuum*, *Glycine*

A

```
  1  GTGGTGCAGAGGCAAGAACCTGCGAGTCACAGAGCCACAGTTTCAAGGGGCCATGTGTTG
  1    G  A  E  A  R  T  C  E  S  Q  S  H  S  F  K  G  P  C  V  G

 61  GCGATACCAACTGCGCCTCCGTGTGCCAGACTGAAGGTTTCATTGGCGGCGATTGTCGTG
 21    D  T  N  C  A  S  V  C  Q  T  E  G  F  I  G  G  D  C  R  G

121  GCCTTCGCCGCCAATGTTTTTGCACTAGAAACTGCTAGAAGAAAGTTTCTAAATGATCTT
 41    L  R  R  Q  C  F  C  T  R  N  C

181  TTTAACTGTCTATGTATTTATTTCCTTGTTAAAATATCTAATGATAAATAATACTGCTCT
241  ATCAATAAAAAAAAAAAAAAAAAAA
```

B

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                    1                                                  50
S.tuberosum CP34    ..............................GAEARTCESQSHSFKGPCVGDT
P.hybrida PPT       MGRSIRLFATFFLIAMLFLSTE.MGPMTS-----------R-H-T--RES
A.thaliana GBGY6    MKLSMRLISAVLIMFMIFVATG.MGPVTV.----------R---T--SAS
B.rapa BIF25        MKLSMRLISAVLLLFMIFVATG.MGPVTV.-------K--R---T--SS-
C.annuum JL-2       MAGFSKVIATIFLMMMLVFATDMM.....---KI--AL-GN---L-LSSR
G.max PIN1          MSRSVPLVSTICVLLLLLVATEMMGPTMV-----------R-----LS--
S.tuberosum P322    ....MRFFATFFLLAMLVVATK.MGPMRI----H---L--R-----TR-S
N.tabacum FST       ......MARSLCFMAFAILAMMLFVAYEVQARE.-KTE-NT-P-I-ITKP
L.esculentum TPP3   ......MARSIFFMAFLVLAMMLFVTYEVEAQQI-KAP-QT-P-L-FM-S
                                                     *   *  * * *

                    51                                                 100
S.tuberosum CP34    NCASVCQTEGFIGGDCRGLRRQCFCTRNC.....................
P.hybrida PPT       --------------N--AF--R-------.....................
A.thaliana GBGY6    ---N--HN---V--N---F--R-----H-.....................
B.rapa BIF25        --GN--HN---G--K---F-VR-Y---H-.....................
C.annuum JL-2       D-GN--RR---TS-V---FPLK---RKPGA....................
G.max PIN1          --G---R--R-T--H---F--R----KH-.....................
S.tuberosum P322    ------E--R-S--N-H-F--R----KP-.....................
N.tabacum FST       P-RKA-IS-K-TD-H-SK-L-R-L--KP-VFDEKMIKTGAETLVEEAKTL
L.esculentum TPP3   S-RKY-IK-K-T--H-SK-Q-K-L--KP-VFD.KISSEVKATLGEEAKTL
                     *   *  * *  * *      * *

                    101      112
S.tuberosum CP34    ............  51
P.hybrida PPT       ............  78
A.thaliana GBGY6    ............  77
B.rapa BIF25        ............  77
C.annuum JL-2       ............  75
G.max PIN1          ............  79
S.tuberosum P322    ............  74
N.tabacum FST       AAALLEEEIMDN 105
L.esculentum TPP3   SEVVLEEEIMME 105
```

Figure 5 Sequence analysis of cp34

A. Nucleotide sequence of the cp34 cDNA and the deduced amino acid sequence. The nucleotide and amino acid sequences are numbered at the left. The stop codon is shown in boldface. The conserved cysteine residues are shown in boldface. B. Alignment of the deduced amino acid sequence of CP34 with

γ-thionin-like proteins The deduced γ-thionin-like proteins of *Petunia inflata* (PPT, Karunanandaa *et al*, 1994), *Arabidopsis thaliana* (GBGY6, Accession number X69139), *Brassica rapa* (BIF25, Accession number L31937), *Capsicum annuum* (JL-2, Accession number X95730), *Glycine max* (PIN1, Accession number U12150), *Solanum tuberosum* (P322, Stiekema *et al*, 1988), *Nicotiana tabacum* (FST, Gu *et al*, 1992) and *Lycopersicon esculentum* (TPP3, Milligan and Gasser, 1995) are aligned using the PILEUP programme of the University of Wisconsin Computer Group (Devereux *et al*, 1984) Sequence positions are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence Amino acids identical to those in CP34 are indicated by dashes Dots represent gaps Residues that are completely identical among γ-thionin-like proteins are marked by an asterisk

max, *S tuberosum* (Stiekema *et al*, 1988), *N tabacum* (Gu *et al*, 1992) and *Lycopersicon esculentum* (Milligan and Gasser, 1995) The identity between the CP34 and the γ-thionins isolated from the endosperm of wheat and barley ranged from 51% to 53%, respectively (Colilla *et al*, 1990, Mendez *et al*, 1990)

To examine the spatial expression of the *cp34* gene in more detail, cp34 cDNA was used as a probe in a Northern blot analysis of RNA from various tissues of potato As shown in Figure 6, a mRNA species of 0 45 kb was only detected in pistils which indicates that *cp34* is a pistil-specific gene in potato

The cp37 gene is specifically expressed in the pistil

Analysis of the spatial expression of the *cp37* gene revealed that *cp37* transcripts were only present in pistils No hybridisation signal was detected in the other flower tissues or vegetative parts of the potato plant (Figure 6) The cp37 cDNA comprises 290 bp and sequence analysis revealed no open reading frame (Figure 7) Furthermore, sequence database searches did not result in any homology of the cp37 cDNA clone with other sequences This indicates that cp37 is a new pistil-specific cDNA clone

The cp71 cDNA shows homology to hormone induced proteins

The cp71 cDNA was shown by sequence analysis to be 277 bp in length and to contain an open reading frame encoding a 85 amino acid polypeptide when starting from the first nucleotide No starting methionine is present and the length of the cDNA is much shorter than

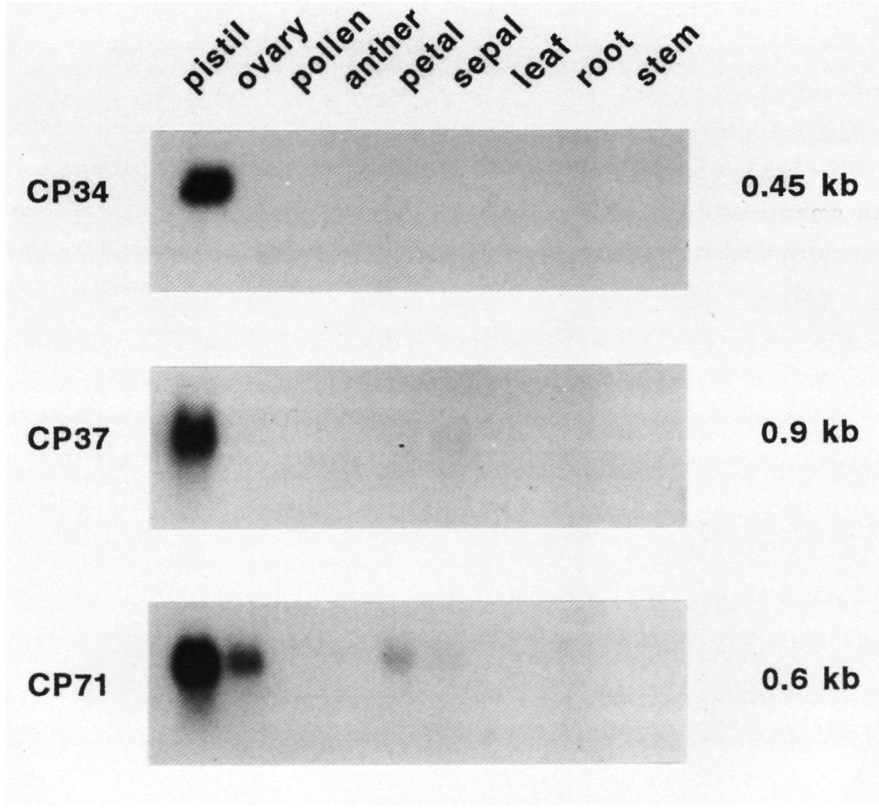

Figure 6 Northern blot analysis of expression of three different genes detected by the cold-plaque technique

Expression of the *cp34*, *cp37*, and *cp71* genes in various potato organs. Total RNA (10 µg per lane) was isolated, fractionated by agarose/formaldehyde gel electrophoresis and transferred to nylon membranes. After hybridisation to the various cDNA probes, the blots were washed in 0.5x SSC, 0.1% SDS at 55°C and used for autoradiography. The size (kb) of the hybridising band is indicated.

the size of the mRNA (0.6 kb) estimated from Northern blot analysis (Figure 8A and Table 1). The CP71 protein contains many cysteine residues (14%). Comparison of the predicted CP71 protein with sequence databases showed the highest homology with several proteins inducible by gibberellins and auxins. The length of the cDNAs, corresponding to these hormone-induced

```
  1 ctcaaaccaaacaaatttgctggaggaacatttaatactgggctgattttaatcccattg
 61 atgtaacctggaactttcctcaatggaggtttctgataatgtccgttcgaagaagaaaca
121 gaggatgatgatttatggaatgatgtttgtgtttcatgtttggagttgaaccagttgaat
181 tgaattacttcgcatagaattggtaaaggacataaagttcttacgtaactacatgtacta
241 ctggtactattactacgcttgaatctccaaatcactttgagctctgtttt
```

Figure 7 Nucleotide sequence of the cp37 cDNA
The nucleotide sequence is numbered at the left.

genes, ranged from approximately 500 to 700 bp and all cDNAs contain a long 3' untranslated region of 150 to 350 bp without any poly(A)$^+$ tail. However, the 3' untranslated region of cp71 consisted of only 21 bp (Figure 8A).

The deduced CP71 protein is presented in Figure 8B and compared with the six most related amino acid sequences of gibberellin-induced GASA proteins of *A. thaliana* (Herzog *et al.*, 1995), the auxin-induced RSI-1 protein from tomato (Taylor and Scheuring, 1994), the *P. hybrida* GIP1 protein and the gibberellin and auxin regulated GAST1 protein from tomato (Shi *et al.*, 1992). For all protein sequences homology was restricted to the C-terminal region of 60 amino acids, which displays a total of 24 conserved residues, including 12 cysteines (Figure 8B). Sequence identity scores between the C-terminal region of CP71 and the aligned proteins ranged from 45% for GAST1 and RSI-1 from tomato to 80% for GASA1 from *A. thaliana*. No significant homology was found with the N-terminal part of the CP71 protein.

Cp71 is predominantly expressed in the carpel

After initial expression analysis (Table 1), the tissue-specific expression pattern of the *cp71* gene was further determined by Northern blot analysis of total RNA of several tissues (Figure 6). The cp71 cDNA strongly hybridised to a single mRNA transcript present in pistils and in ovaries. A weak signal was also detected in petals, thus confining the *cp71* expression to flowers. This expression pattern of *cp71* is comparable to the expression of the *gasa1* gene from *A. thaliana*, transcripts of which were mainly detected in flower buds and green siliques (Herzog *et al.*, 1995). Cp71 mRNA transcripts were not present in tissues outside the flower.

A

```
  1  CAAGTCCAATCTATTCAGACCGATCAAGTGACCAGCAATGCTATTTCTGAAGCCGCTTAT
  1  Q  V  Q  S  I  Q  T  D  Q  V  T  S  N  A  I  S  E  A  A  Y

 61  TCCTACAAGAAAATTGACTGTGGGGGAGCTTGTGCAGCAAGGTGCCGATTATCATCAAGG
 21  S  Y  K  K  I  D  C  G  G  A  C  A  A  R  C  R  L  S  S  R

121  CCAAGATTGTGTAATAGGGCATGTGGAACTTGTTGTGCTAGATGCAACTGTGTTCCTCCT
 41  P  R  L  C  N  R  A  C  G  T  C  C  A  R  C  N  C  V  P  P

181  GGTACTTCTGGCAACACTGAGACTTGCCCTTGCTATGCCAGTTTGACTACTCATGGCAAC
 61  G  T  S  G  N  T  E  T  C  P  C  Y  A  S  L  T  T  H  G  N

241  AAACGTAAATGCCCTTAATTTTCTTCTAAAATATCCTCT
 81  K  R  K  C  P
```

B

```
                    1                                                  50
S.tuberosum CP71    ................QVQSIQTDQVTSNAISE.........  ....A
A.thaliana GASA1    MAISKALIASLLISLLVL-LV-A-VES-QKKNG.................
A.thaliana GASA2    ....MAVFRSTLVLLLIIVCLT-YELHVH-ADG.............AKV
A.thaliana GASA3    ....MAIFRSTLVLLLILFCLT-FELHVH-AED.........    .SQV
L.esculentum RSI-1  .MAKSGYNASFLLLISMFLILL-FSNVVEGY.............  ......
P.hybrida GIP1      ....MAGKLSIVLFV.LLVVLLAQNQV-R-KMVLDSKVQRRGNDQIYGVS
L.esculentum GAST1  ....MAGKMSIVLFV.LLVVFL-QNQV-R-NIMRDEQQQQQRNNQLYGVS

                    51                                                 100
S.tuberosum CP71    AYSYKKIDCGGACAARCRLSSRPRLCNRACGTCCARCNCVPPGTSGNTET
A.thaliana GASA1    ..YA------S--V---R--------H--------Y---------Y--YDK
A.thaliana GASA2    GEGVV------R-KD--SK---TK--L---NS--S-------------HL
A.thaliana GASA3    GEGVV------R-KG--SK----N--L---NS--Y---------A--HHL
L.esculentum RSI-1  .NKLRPT--KPR-TY--SAT-HKKP-MFF-QK---T-L---K-VY--KQS
P.hybrida GIP1      QG-LHPQ--QPK-TY--SKT-FKKP-MFF-QK---K-L---A--Y--KQ-
L.esculentum GAST1  EGRLHPQ--QPK-TY--SKT-YKKP-MFF-QK---K-L---A--Y--KQS
                          **   *  **   *    *   *  **  * *** *  **

                    101           117
S.tuberosum CP71    CPCYASLTTHGNKRKCP  85
A.thaliana GASA1    -Q---------GR----  98
A.thaliana GASA2    ------I----GRL---  99
A.thaliana GASA3    ------I--R-GRL---  99
L.esculentum RSI-1  ----NNWK-QEG-P---  96
P.hybrida GIP1      ----NNWK-KEGGP--- 112
L.esculentum GAST1  ----NNWK-KRGGP--- 112
                    * **    *     ***
```

Figure 8 Sequence analysis of cp71

A. Nucleotide sequence of the cp71 cDNA and the deduced amino acid sequence The nucleotide and amino acid sequences are numbered at the left The stop codon is shown in boldface. The conserved C-terminal region of 60 amino acids is underlined B. Alignment of the deduced amino acid sequence of CP71 with hormone-regulated proteins The deduced hormone-regulated proteins of *Arabidopsis thaliana* (GASA1,2,3, Herzog *et al*, 1995), *Lycopersicon esculentum* (RSI-1, Taylor and Scheuring, 1994),

Petunia hybrida (GIP1, Accession number X87225) and *Lycopersicon esculentum* (GAST1, Shi *et al*, 1992) are aligned using the PILEUP programme of the University of Wisconsin Computer Group (Devereux *et al*, 1984) Sequence positions are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence Amino acids identical to those in CP71 are indicated by dashes Dots represent gaps Residues that are completely identical among the hormone-regulated proteins are marked by an asterisk

Discussion

For effective reproduction, the pistil has to provide gene products that contribute to a suitable environment for successful pollen germination, pollen tube growth and finally seed set However, we may expect that some of the gene products will not be abundantly present To investigate gene products present in pollinated pistils we have set out a cold-plaque screening procedure to isolate cDNAs corresponding to mRNAs not present at an abundant level in pollinated pistils of *Solanum tuberosum* (Hodge *et al*, 1991) However, by this protocol we isolated cDNA clones corresponding to genes which proved to be expressed at a relatively high level (Figure 3A, Figure 6, chapters 5 and 6) Six out of 33 cDNA clones isolated by this procedure correspond to genes expressed in the pistil (Table 1) This indicates that the cold-plaque screening was successful, since 18% of the tested clones meet the expected expression pattern

A potato alcohol dehydrogenase-like gene is predominantly expressed in pistils

Alcohol dehydrogenases in plants are cytosolic enzymes which catalyse the oxidation of various alcohols to aldehydes or ketones, utilising NADH as a cofactor The presumed physiological role for ADH is its reverse reaction, in which ADH reduces acetaldehyde to ethanol, regenerating NAD^+, thereby allowing glycolysis to maintain sufficient levels of cellular ATP during periods of oxygen deprivation (Gregerson *et al*, 1991) The predicted CP67 protein has 45% amino acid identity with long-chain zinc-containing alcohol dehydrogenases

This similarity is significantly lower than the one found among the previously identified plant ADHs which range from 71% to 97% (Sun and Plapp, 1992). Other exceptions are the two described ADH3 proteins from tomato which share only 56% to 59% amino acid identity with other plant ADHs (Ingersoll *et al.*, 1994).

The *cp67* gene is predominantly expressed in pistils. This is in contrast with the expression of the other *adh* genes which are, in plants grown under normal, aerobic conditions, only transcribed in pollen (Bucher *et al.*, 1995; Schwartz, 1971). *Adh* genes are expressed at high levels in all tissues only after oxygen deprivation or elicitor treatment (Freeling and Bennett, 1985; Matton *et al.*, 1990; Xie and Wu, 1989). The expression of the *cp67* gene in pistils, together with the absence of ADH activity in unpollinated pistils, suggests that cp67 mRNA is not translated in non-pollinated pistils or encodes a protein not involved in ADH metabolism.

Pollen tube growth induces adh *gene expression and ADH enzyme activity*

The presence of ADH activity and the accumulation of sth-11 mRNA in pollinated pistils, as compared to unpollinated pistils, demonstrate that both *adh* gene expression and ADH enzyme activity are induced in pistils upon pollen tube growth (Figure 4). Indirect evidence for ADH activity during *in vitro* pollen tube growth was obtained by the accumulation of ethanol to high levels in germination medium in the presence of carbohydrates (Bucher *et al.*, 1995). Furthermore, Bucher *et al.* (1995) concluded that during pollen tube growth respiration is insufficient to fulfil the requirement for energy, and hence fermentation is used as an accessory energy-generating pathway. The ethanol synthesised by the pollen tubes grown *in vivo* would be remetabolised by the pistil through the action of ADH (Bucher *et al.*, 1995). However, the ADH activity is not essential for plant reproduction, because mutant plants that do not express the *adh* gene are normally fertile (Freeling and Bennet, 1985; Wisman *et al.*, 1993).

A comparison between transcript levels and ADH activity indicates that the ADH activity observed in potato pistils is probably regulated at the transcriptional level, as it was also described for other plant species (Kadowaki *et al.*, 1988; Rousselin *et al.*, 1994; Xie and Wu, 1989). In both pollen and complete anthers of potato ADH activity was detected. However, the *sth-11* gene was predominantly expressed in whole anthers whereas in mature pollen only a faint hybridisation signal was detected (Figure 4). The anther-specific *adh3* gene from tomato

(Ingersoll *et al.*, 1994) showed only expression in complete anthers and not in mature pollen of potato (data not shown). This difference between *adh* gene expression and ADH enzyme activity in pollen has also been described for *P. hybrida* and suggests, but does not prove, the deposition of anther derived ADH proteins onto pollen (Gregerson *et al.*, 1991). However, the predominant expression of an *adh* gene in mature pollen of *N. tabacum* (Bucher *et al.*, 1995) indicates that the possibile expression of an *adh* gene during early stages of potato pollen development can not be excluded .

A γ-thionin-like gene is specifically expressed in the pistil

The predicted CP34 protein belongs to a recently described family of γ-thionin-like proteins. Most γ-thionin-like proteins were described to be proteinase inhibitors, because of their homology with p322 of *S. tuberosum* which was formerly reported as a proteinase inhibitor homologue. More recently it has been shown that p322 displays more homology with γ-thionins (Karunanandaa *et al.*, 1994; Stiekema *et al.*, 1988). Thionins are a group of low molecular weight proteins assumed to function in plant defence against pathogens (Florack and Stiekema, 1994), since *in vitro* they are toxic to bacteria, fungi and yeast, which results from the destruction of biological membranes (Mendez *et al.*, 1990). The original γ-thionins, a special class of thionins, were firstly detected in wheat and barley endosperm (Colilla *et al.*, 1990; Mendez *et al.*, 1990). The homology between γ-thionin-like proteins and the γ-thionins of wheat and barley suggests that they may have the same toxic effect on bacteria and fungi. This homology and the presence of γ-thionin-like cp34 and ppt transcripts in pistils of both *P. inflata* and *S. tuberosum* suggest a possible role in the defence of the pistils against pathogen infection. Such a defence mechanism is necessary in the pistil, as due to the penetration of the non-aseptic pollen tubes and the presence of macromolecules required for supporting pollen tube growth, the pistil is vulnerable to pathogen infection and has therefore to be protected. The production of defence molecules is expected upon pollen tube growth and may be more effective if they are already present before pollination. The *cp34* gene can be added to a growing list of pistil-expressed genes that encode defence related proteins (see also chapter 1 and 3).

Cp71, predominantly expressed in carpels, is homologous to hormone-induced genes

The predicted CP71 protein has homology to a small family of hormone-induced proteins. Members of the family are the GASA proteins from *A. thaliana* (Herzog *et al.*, 1995), a GIP1 protein from *P. hybrida* (Ben-Nissan, unpublished) and from tomato the RSI-1 and GAST1 proteins (Shi *et al.*, 1992; Taylor and Scheuring, 1994). The mutual homology is located in the conserved C-terminal domain, with 12 cysteine residues at conserved positions suggesting a globular conformation through disulphate bridges (Herzog *et al.*, 1995). This C-terminal domain is transcribed, by the last exon of the GASA, RSI-1 and GAST1 genes (Herzog *et al.*, 1995; Shi *et al.*, 1992; Taylor and Scheuring, 1994).

The isolated cp71 cDNA lacks, together with the homologous sequences presented in Figure 8, a poly(A)$^+$ tail. Transcripts with short or even without poly(A)$^+$ tails are normally subjected to degradation. In pollinated pistils of tobacco poly(A)$^+$ tails of tts transcripts are shortened, but these transcripts are maintained at high levels. This suggests that specific transcripts can be protected from degradation and can be present with short or probably without poly(A)$^+$ tails (Wang *et al.*, 1996).

The expression of *cp71* in the carpel and petal is comparable to the expression of the *gasa1*, *gasa4* and *gast1* genes which are expressed in whole flower buds (Herzog *et al.*, 1995; Shi *et al.*, 1992). The *gip1* gene is also expressed in flowers, because it was isolated from a *P. hybrida* corolla cDNA library (Ben-Nissan, unpublished). The less homologous *rsi-1* gene is only expressed in lateral root primordia (Taylor and Scheuring, 1994). The predominant expression of the *cp71* gene in the carpel suggests a possible function during pistil development or pollen tube growth. The possibility that the expression of *cp71* in the carpel is under hormonal control, just as the other homologous genes, has to be further investigated.

Acknowledgements

We thank Dr. N.M. Brisson (Université de Montréal, Montréal, Canada) for providing the *S. tuberosum* sth-11 clone, Dr. M.R. Hanson (Cornell Univerisity, Ithaca, USA) for providing the *L. esculentum* adh3 clone and Theo Geurts and Gerard van der Weerden for culturing the plants. Manuscript editing was done by Dr. A.F. Croes. This work was supported by the EC Bridge programme BIOT 900172.

Materials and Methods

Plant material

Potato plants (*Solanum tuberosum*) were grown in a climate chamber at 20°C under a light/dark regime of 16/8 h. Characteristics of plants were described elsewhere (chapter 2, Van Eldik *et al.*, 1995). Pollinations were carried out with mature pollen collected from anthers at anthesis and applied to the stigma of flowers with anthers just before anthesis. For anoxia studies, leaves were placed under water for 18 h. All plant material was collected from mature flowers and plants.

Nucleic acid methods

Total RNA was isolated using the method of Frankis and Mascarenhas (1980) with slight modifications (Goldberg *et al.*, 1981). The gel blot analyses were performed as described in chapter 2. For the detection of the various transcripts the cp34, cp37, cp67, cp71 and sth-11 cDNAs (Matton *et al.*, 1990) from *S. tuberosum* were used. Furthermore, the adh3 cDNA clone of *L. esculentum* was used to detect the anther-specific adh transcripts (Ingersoll *et al.*, 1994). Isolation of plasmid DNA, subcloning and restriction analysis were performed using standard procedures (Sambrook *et al.*, 1989). Nucleotide sequence analysis was performed using the T7 DNA polymerase sequencing system of Pharmacia. Both nucleotide and deduced protein sequences were analysed using the University of Wisconsin Computer Group

programmes (Devereux *et al.*, 1984)

Isolation of cold-plaque cDNA clones

A cDNA library of pollinated pistils (chapter 2, Van Eldik *et al.*, 1995) was screened using the cold-plaque method as described by Hodge *et al* (1991). A ^{32}P-labelled single-stranded cDNA probe, prepared from poly(A)$^+$ mRNA of pollinated pistils, was used. This was the same material from which the cDNA library was constructed. Approximately 10 000 pfu were screened at 45°C in 4x SETS (1x SETS is 0.15 M NaCl, 0.02 M TRIS-HCl pH 7 8, 1 mM EDTA), 5x Denhardt's (1x Denhardt's is 0.02% Ficoll, 0 02% PVP and 0 02% BSA), 0 1% SDS and 75 μg/ml denatured herring sperm DNA.

The isolated cp67 cDNA clone was not full-sized. Therefore, the 5'-Amplifinder RACE kit from Clontech was used to isolate the 5' end of the cp67 cDNA clone Two nested internal primers were designed (cp672, 5'-ATTGGACTTCCCTGACTTGC-3', cp673, 5'-GGGCATTCTTTGCATTCTCC-3') The primer cp672 was used to synthesize cDNA and the primer cp673 was used for PCR amplification of the 5' cDNA end according to the protocols of the manufacturer The PCR fragments were cloned with the TA Cloning System from Invitrogen into the pCRII vector

Native polyacrylamide gel electrophoresis

Gels containing 7.5% polyacrylamide were prepared in 1x TBE (90 mM TRIS-Borate, 1 mM EDTA). Electrophoresis was performed at room temperature at 200 V in 1x TBE

ADH activity assay by gel staining

The different potato tissues were powdered separately in liquid nitrogen using a mortar and pestle and then mixed with extraction buffer (100 mM TRIS-HCl pH 8 0, 5 mM DTT, 2% PVPP, 0.1% β-mercaptoethanol) at 4°C Native polyacrylamide gel electrophoresis was performed with each lane containing 10 µg protein The gels were soaked in an ADH activity staining solution of 300 µg/ml NAD$^+$, 200 µg/ml nitroblue tetrazolium, 100 µg/ml phenazine methanosulfonate, 1 mM TRIS-HCl pH 7 5 and 10% ethanol for 30 min at 37°C in the dark (Gregerson *et al.*, 1991) After staining the gels were washed in distilled water

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CHAPTER 5

Regulation of flavonol biosynthesis during anther and pistil development, and during pollen tube growth in *Solanum tuberosum*

submitted to *Plant Journal*

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Abstract

The regulation of flavonol biosynthesis was studied in anthers and pistils of *Solanum tuberosum*. Flavonols are essential for functional pollen tube growth in a number of species. Flavonol accumulation in whole anthers started at the unicellular stage of pollen development and continued until pollen maturity. A cDNA clone encoding flavonol synthase (FLS) was isolated. *Fls* gene expression was detected in pistils, anthers, petals and ovaries, the organs in which flavonols are accumulating. Fls transcripts were present in unicellular and bicellular pollen, but not in mature pollen. The expression patterns of three genes encoding enzymes involved in the flavonoid biosynthetic pathway, chalcone synthase (*chs*), flavanone-3-hydroxylase and *fls* were analysed in developing anthers and pistils. Only chs transcripts accumulated concomitantly with the flavonols in anthers. In pistils of potato, pollen tube growth induced an increase in *fls* gene expression that, unlike the situation in pollinated pistils of *Petunia*, did not result in an increased flavonol content. We conclude that (1) flavonol biosynthesis in anthers is probably initiated by the expression of the *chs* gene and (2) that flavonol accumulation in pistils upon pollen tube growth is not an universal phenomenon.

Introduction

Pollination in higher plants starts with the landing of the pollen, shed from the anther, on the stigma where the pollen germinates The protruding pollen tubes grow through the stigma and transmitting tissue of the style towards the ovary where the two sperm cells fuse with the egg and the central cell nuclei, forming the zygote and the endosperm (Derksen *et al* , 1995, Esau, 1977)

When the pollen is released from the anther, it consists of products synthesised by the pollen itself and material produced by the tapetum (Mascarenhas, 1990) The growth of the pollen tubes towards the ovary is influenced by compounds present in the pollen and pistil, and by

products synthesised through interactions between the pistil and the growing pollen tubes (Cheung *et al.*, 1995; Herrero and Arbello, 1989; Mascarenhas, 1993; Vogt *et al.*, 1994).

Flavonols, a special class of flavonoids, are among the low molecular weight substances involved in the pollination process (Koes *et al.*, 1994). These flavonols are present in both pollen and pistil. In *in vitro* cultured pollen of tobacco and *Brassica oleracea*, development and germination are enhanced by the addition of flavonols to the culture medium (Sedgley, 1975; Ylstra *et al.*, 1992). Flavonol accumulation is induced by pollination or by wounding of other flower organs in pistils of *Petunia*, but not in maize (Vogt *et al.*, 1994). Pollen from flavonol-deficient maize and *Petunia* plants is unable to form functional pollen tubes, which makes the plants self-sterile. This deficiency can be complemented by adding flavonols to the pollen or to the stigma (Mo *et al.*, 1992; Pollak *et al.*, 1995; Van der Meer *et al.*, 1992; Ylstra *et al.*, 1994). However, the role of flavonols during pollen tube growth is not undisputed because the flavonol-deficient *Arabidopsis thaliana* tt4 mutant exhibits normal pollen tube growth and seed set (Shirley *et al.*, 1995; Ylstra, 1995; Burbulis *et al.*, 1996).

In flowers, flavonols accumulate in petals, pistils, ovaries and anthers (Davies *et al.*, 1993; Koes *et al.*, 1990; Pollak *et al.*, 1993; Ylstra, 1995). According to the current hypothesis, flavonols are synthesised in the tapetum and deposited on the developing pollen after tapetal breakdown at the end of anther development. Their uptake by the pollen cytoplasm results in the presence of flavonols inside the grains (Beerhues *et al.*, 1989; Vogt and Taylor, 1995; Wiermann and Vieth, 1983; Ylstra, 1995). However, there is accumulating evidence that genes encoding enzymes of the flavonoid biosynthetic pathway are also expressed in pollen during early development, which opens the possibility that flavonols are produced in developing microspores as well (Hauffe *et al.*, 1993; Ohl *et al.*, 1990; Shen *et al.*, 1992; Van Tunen *et al.*, 1990).

Flavonols are produced via a branch of the flavonoid biosynthetic pathway (Figure 1). Chalcone synthase (CHS) catalyses the initial step in flavonoid biosynthesis and forms chalcones. The chalcones are isomerised by chalcone isomerase (CHI) into flavanones. Hydroxylation of flavanones leads to the production of dihydroflavonols by flavanone-3-hydroxylase (F3H). The conversion of dihydroflavonols in flavonols is catalysed by an enzyme, flavonol synthase (FLS) (Britsch *et al.*, 1981). In developing flower buds of *Petunia*, *Dianthus*

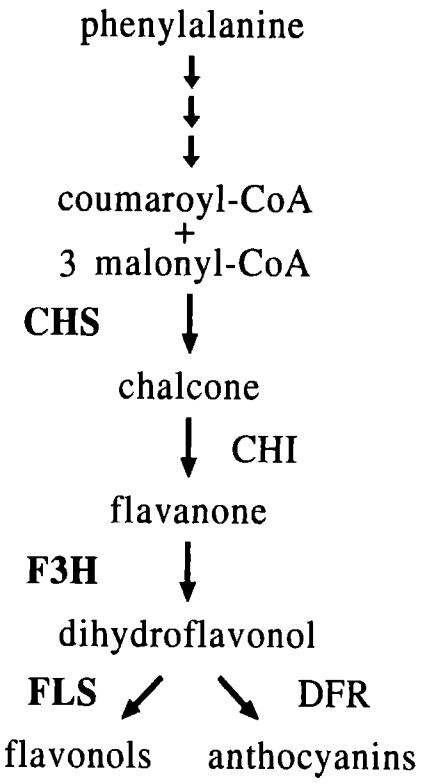

Figure 1 The flavonoid biosynthetic pathway

The enzymes corresponding to the genes investigated are shown in boldface letters CHS, chalcone synthase, CHI, chalcone isomerase, F3H, flavanone-3-hydroxylase, FLS, flavonol synthase, DFR, dihydroflavonol 4-reductase dioxygenase

caryophyllus and *Matthiola incana*, the FLS enzyme activity is temporally regulated (Forkmann *et al* , 1985, Spribille and Forkmann, 1984, Stich *et al* , 1992) The corresponding fls cDNA clone has been isolated from *Petunia* (Holton *et al* , 1993)

The role of flavonols in pollen tube growth is thus a matter of dispute The kinetics of flavonol accumulation and the developmental expression patterns of genes encoding enzymes of the flavonoid biosynthetic pathway in anthers and in pistils, before and after pollination, will give more insight in the role of flavonols during the pollination process These enzymes are necessary for flavonol biosynthesis, so the expression of all the corresponding genes is expected in case of flavonol accumulation The expression levels of these genes might indicate

which enzyme will be rate-limiting for flavonol biosynthesis, on the assumpation that the enzyme activity more or less mirror the expression levels. At the moment, nothing is known about the role of flavonols in pollen and pistil fertility of potato. An fls cDNA clone was isolated from *Solanum tuberosum* and the developmental expression of three genes (*fls*, *chs* and *f3h*) encoding enzymes of the flavonoid biosynthetic pathway was analysed and correlated with flavonol concentration. *Fls* was investigated because it encodes the enzyme directly producing flavonols. We selected *chs* because it catalyses the initial step in flavonoid production and because a blocked *chs* gene resulted in male-sterility in *Petunia* (Van der Meer *et al.*, 1992). *F3h* was chosen because it encodes the enzyme that produces the direct substrate for the flavonol synthase enzyme, and regulates flavonol production in maize anthers (Deboo *et al.*, 1995). Only chs transcript accumulation correlated with the accumulation of flavonols in the anthers. The *fls* gene was found to be transcribed in pollen during early development. In contrast to *Petunia*, no increase in the flavonol content was detected in potato pistils during pollen tube growth, although fls transcripts accumulated in pollinated pistils.

Results

Flavonol accumulation during anther development and pollen tube growth

The anthers of potato contain several flavonols. Kaempferol was the most abundant flavonol in hydrolysed extracts, furthermore, low amounts of quercetin and traces of myricetin were detected. The same flavonols were detected in mature pollen. In unhydrolysed extracts no flavonol aglycones were detected, indicating that all endogenous flavonols are present in a conjugated form (data not shown). If flavonols are important in pollen maturation and pollen tube growth, they would be expected to accumulate towards the end of anther development. No flavonols were detected in anthers of 3-4 mm flower buds (tetrad stage) but the accumulation started in anthers at the unicellular pollen stage (5-6 mm) and increased towards pollen maturity (Figure 2A). The total kaempferol content of mature anthers was approximately 900 pmol mg^{-1} fresh weight.

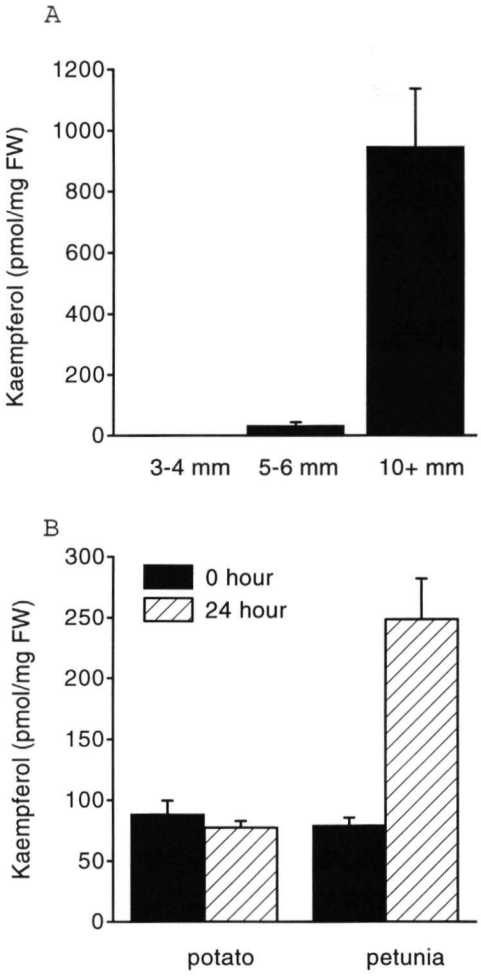

Figure 2 Accumulation of kaempferol during anther development and pollen tube growth in the pistil

A. Anthers of 3-4 mm, 5-6 mm and 10+ mm flower buds were extracted in ethanol and hydrolysed. Kaempferol aglycones (pmol/mg fresh weight) were detected by analytical HPLC (λ = 365 nm). Values are the means of two replicates. Error bars represent SE. B. Pistils of *Solanum tuberosum* and *Petunia hybrida* were extracted in ethanol and hydrolysed at 0 h and 24 h after pollination. Kaempferol aglycones were detected by analytical HPLC (λ = 365 nm). Values are the means of at least two replicates. Error bars represent SE.

Because of the reported necessity of flavonols during the process of pollen tube growth in *Petunia*, we compared the flavonol content of pollinated and unpollinated pistils of potato and *Petunia*. Flavonols were extracted from pistils at 0 and 24 h after pollination and their amount quantified (Figure 2B). In potato pistils, kaempferol content did not change significantly during the first 24 h after pollination. In contrast, the kaempferol content of *Petunia* pistils had increased more than threefold in the same period. Similar results were obtained for quercetin (data not shown).

A

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   1 AAAAGAATGAAAACAATTCAAGGTCAGAGTGCAACAACAGCCTTGACGATGGAGGTGGCA
   1       M  K  T  I  Q  G  Q  S  A  T  T  A  L  T  M  E  V  A

  61 AGGGTCCAAGCAATATCGTCAATAACGAAATGCATGGACACAATACCATCAGAATATATT
  19 R  V  Q  A  I  S  S  I  T  K  C  M  D  T  I  P  S  E  Y  I

 121 CGTTCAGAGAACGAACAACCTGCAGCGACAACGCTGCAGGGAGTGGTACTTGAAGTACCA
  39 R  S  E  N  E  Q  P  A  A  T  T  L  Q  G  V  V  L  E  V  P

 181 GTCATCGACATAAGTAATGTCGATGACGACGAAGAAAAGTTAGTGAAAGAAATAGTTGAG
  59 V  I  D  I  S  N  V  D  D  D  E  E  K  L  V  K  E  I  V  E

 241 GCTAGTAAAGAGTGGGGTATTTTTCAAGTGATAAATCATGGGATACCTGATGAAGTGATT
  79 A  S  K  E  W  G  I  F  Q  V  I  N  H  G  I  P  D  E  V  I

 301 GAGAATTTGCAAAAAGTTGGAAAAGAGTTTTTTGAGGAAGTGCCACAAGAGGAAAAAGAA
  99 E  N  L  Q  K  V  G  K  E  F  F  E  E  V  P  Q  E  E  K  E

 361 TTGATTGCAAAGAAGCCAGGGGCACAAAGTTTAGAAGGATATGGTACTTCTTTGCAGAAG
 119 L  I  A  K  K  P  G  A  Q  S  L  E  G  Y  G  T  S  L  Q  K

 421 GAAATTGAAGGGAAAAAAGGTTGGGTTGATCATTTGTTTCATAAGATTTGGCCTCCTTCT
 139 E  I  E  G  K  K  G  W  V  D  Y  W  H  L  F  H  K  I  W  P

 481 GCTATTAACTATCGTTATTGGCCAAAAAATCCTCCTTCGTACAGGGAAGCAAATGAGGAA
 159 P  S  A  I  N  Y  R  P  K  N  P  P  S  Y  R  E  A  N  E  E

 541 TACGCAAAGTGGCTGCGAAAAGTTGCTGATGGTATATTTAGGAGCTTGTCACTTGGGCTT
 179 Y  A  K  W  L  R  K  V  A  D  G  I  F  R  S  L  S  L  G  L

 601 GGTTTGGAAGGCCATGAAATGATGGAGGCAGCTGGTAGTGAAGACATAGTTTACATGTTA
 199 G  L  E  G  H  E  M  M  E  A  A  G  S  E  D  I  V  Y  M  L

 661 AAGATCAATTATTATCCACCATGCCCAAGGCCTGATTTGGCTCTTGGAGTTGTGGCCCAT
 219 K  I  N  Y  Y  P  P  C  P  R  P  D  L  A  L  G  V  V  A  H

 721 ACAGATATGTCATATATCACCCTTCTTGTCCCAAATGAAGTCCAAGTGTTTAAGGATGGT
 239 T  D  M  S  Y  I  T  L  L  V  P  N  E  V  Q  V  F  K  D  G

 781 CATTGGTATGATGTCAACTACATACCAAATGCTATAATTGTCCACATTGGTGACCAAGTT
 259 H  W  Y  D  V  N  Y  I  P  N  A  I  I  V  H  I  G  D  Q  V

 841 GAGATTCTTAGCAATGGGAAATATAAGAGTGTGTATCATAGGACAACAGTGAACAAGTAC
 279 E  I  L  S  N  G  K  Y  K  S  V  Y  H  R  T  T  V  N  K  Y

 901 AAGACAAGAATGTCATGGCCTGTTTTCTTGGAGCCCTCATCAGAGCATGAAGTTGGGCCA
 299 K  T  R  M  S  W  P  V  F  L  E  P  S  S  E  H  E  V  G  P

 961 ATCCCTAACTTAATTAATGAGGCCAACCCACCCAAATTCAAGACCAAGAAGTACAAAGAT
 319 I  P  N  L  I  N  E  A  N  P  P  K  F  K  T  K  K  Y  K  D

1021 TATGTCTATTGTAAGCTTAACAAACTTCCTCAGTGAAGAATTATGGGGTTAATTCCTCAG
 339 Y  V  Y  C  K  L  N  K  L  P  Q

1081 ATGGTCACTCAACTATCAGTCTTCAGAAAGTCACTCACATGGTGACTTTTTAGTGAAAAT
1141 TAAAAGTTGAGTGACTTTTTGAGGCAAAAGTGGATAGTTGAGTGACCTTATGAGGTATTA
1201 ACCCAAGAATTATGTATCCCATTAATAATTAGCTTGTAACTTCATCAGTATTATGGTTTG
1261 TTGTATTATTATCAAAACATATATATTAACACTTAAGAACTTTTTGCTTAATTTTAAGAT
1321 GTACGATACAAGCTAATGATGAATTGATGATGACTTATATTAATACAAACATAAGCGTAA
1381 AAAAAAAAAAAAAAAAAAAAAAAAAAA
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B
                    1                                                  50
S.tuberosum FLS     MKTIQGQSATTALTMEVARVQAISSITKCMDTIPSEYIRSENEQPAATTL
P.hybrida FLS       ---A--V---..------------LS----------------------
E.grandiflorum FLS  .............---Q---E-A-L.-VI----A----------VS.-V
                               *** *** * *  *  **** **********   *

                    51                                                 100
S.tuberosum FLS     QGVVLEVPVIDISNVDDDEEKLVKEIVEASKEWGIFQVINHGIPDEVIEN
P.hybrida FLS       H----Q-----LR..-P--N-M--L-AD---------L--------A-AD
E.grandiflorum FLS  H----E.----LS..-S--K-I-GLVSE----------V-----N---RK
                     ****  ****    * ** * *     *********  ***** * *

                    101                                                150
S tuberosum FLS     LQKVGKEFFEEVPQEEKELIAKKPGAQSLEGYGTSLQKEIEGKKGWVDHL
P.hybrida FLS       ----------H----------T--SNDI----------V----------
E.grandiflorum FLS  --.--------.L----------PE-S--I-----R----VD---------
                    ** *** ***  **********  *    ***** ****  *********

                    151                                                200
S.tuberosum FLS     FHKIWPPSAINYRYWPKNPPSYREANEEYAKWLRKVADGIFRSLSLGLGL
P hybrida FLS       ---------V-------------------G-RM-E-V-R--K--------
E.grandiflorum FLS  -.----------QF------A----------R-QL..............
                    * ******* **  ****** ******** *

                    201                                                250
S.tuberosum FLS     EGHEMMEAAGSEDIVYMLKINYYPPCPRPDLALGVVAHTDMSYITLLVPN
P hybrida FLS       -----I----GDE---L--------------------------I-----
E.grandiflorum FLS  ..................................................

                    251                                                300
S tuberosum FLS     EV...QVFKDGHWYDVNYIPNAIIVHIGDQVEILSNGKYKSVYHRTTVNK
P.hybrida FLS       --QGL----------K-----L---------------------------
E.grandiflorum FLS  ..................................... .....

                    301                                                350
S.tuberosum FLS     YKTRMSWPVFLEPSSEHEVGPIPNLINEANPPKFKTKKYKDYVYCKLNKL
P hybrida FLS       D-----------P---------K-LS----------------------
E.grandiflorum FLS  ..................................................

                    351
S.tuberosum FLS     PQ 349
P hybrida FLS       -- 348
E.grandiflorum FLS  ..
```

Figure 3 Sequence and protein alignment of a *Solanum tuberosum* cDNA clone encoding flavonol synthase

A DNA sequence and predicted amino acid sequence of the *S. tuberosum* stfls cDNA clone The start and stop codon are shown in boldface The two conserved dioxygenase domains are underlined B. Alignment of the deduced flavonol synthase proteins of *Solanum tuberosum*, *Petunia hybrida* and *Eustoma grandiflorum* Gaps in the alignment are indicated by dashes Identical amino acids are represented by an asterisk underneath the alignment Sequence positions are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence.

Cloning and characterisation of flavonol synthase of S. tuberosum

A cold-plaque screening (Hodge *et al.*, 1991) of a cDNA library from pollinated pistils of *S. tuberosum* (chapter 4) resulted in the isolation of cold-plaque clone cp35 encoding a potato flavonol synthase cDNA clone, now designated stfls. The complete sequence of stfls (1.4 kb) contained an open reading frame of 1047 nucleotides and encoded a polypeptide of 349 amino acids (Figure 3A). This resulted in a predicted protein of 40 kDa with a pI of 5.6.

Comparison of the derived potato FLS amino acid sequence with the *Petunia* FLS amino acid sequence (Holton *et al.*, 1993) revealed 85% identity (Figure 3B). A partial amino acid sequence of *Eustoma grandiflorum* FLS (Nielsen *et al.*, 1994) showed 77% identity. The FLS protein of potato contained two conserved dioxygenase domains (Figure 3A). Surprisingly, the complete 3' untranslated region (322 nt) of stfls showed a homology of 73% with the upstream region of a patatin pseudogene (*sa10c*) of potato (Pikaard *et al.*, 1986).

Southern blot analysis of genomic DNA revealed one strong hybridising band, representing a single-copy *fls* gene, and a few faint bands probably corresponding to partly homologous genes (data not shown).

Spatial and developmental expression of flavonol synthase

The expression of the *fls* gene was monitored by Northern blot analysis of RNA from various tissues. Fls transcripts (1.4 kb in size) were prevalent in pistils, petals, and anthers (Figure 4A). Expression was very low in the ovary and absent in mature pollen, sepals and leaves.

The pattern of flavonol accumulation (Figure 2A) indicated that the biosynthesis is temporally regulated in anthers. Therefore, we followed the expression of the *fls* gene during flower development. Fls mRNA was already present in 1-2 mm flower buds and the amount strongly increased in the 5-6 mm flower bud stage (Figure 4B). In 7-9 mm flower buds, the expression was at its maximum and slightly decreased towards flower maturation. These results show that *fls* gene expression is strictly regulated during flower bud development.

However, the results reveal no information on anthers and pistils, because flower buds contain various somatic and germinal organs including the corolla, which may accumulate high levels of flavonols.

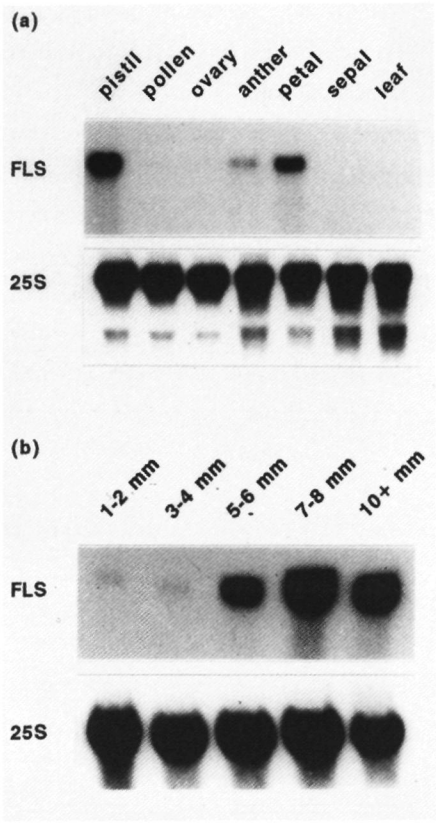

Figure 4 Spatial and developmental expression of the *Solanum tuberosum fls* gene

A. RNA was isolated from various mature organs of *Solanum tuberosum*. Each lane contained 10 µg of total RNA and the membrane was hybridised with ^{32}P-labelled stfls cDNA. After longer exposure a positive signal could also be detected in the ovary. For loading control, *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) was used. B. RNA was isolated from flower buds of different lengths. Each lane contained 10 µg of total RNA and the membrane was hybridised with ^{32}P-labelled stfls cDNA. The various developmental stages, represented by the length of the bud, are described in the Materials and Methods. For loading control, *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) was used.

Expression of the chs, f3h *and* fls *genes during anther and pistil development*

To obtain information on the molecular and developmental regulation of flavonol synthesis in anthers and pistils, we compared the expression of the *fls*, *chs* and *f3h* genes in these organs during flower bud development. The expression was determined in anthers and pistils of 3-4 mm (1) and 5-6 mm (2) flower buds, and of the mature flower (3) (Figure 5). In both anthers and pistils, the level of *fls* transcription was low in the 3-4 mm buds, whereas at the 5-6 mm

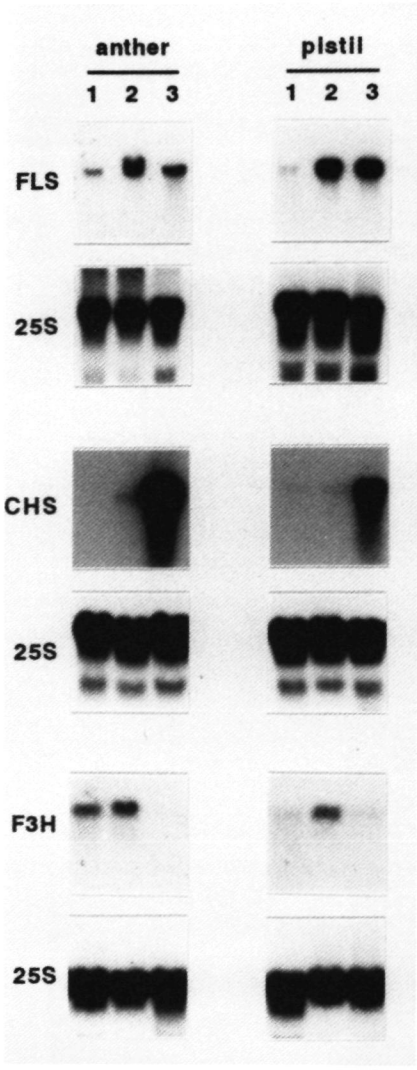

Figure 5 Northern blot analysis of developmental expression of the *fls*, *chs* and *f3h* genes in anthers and pistils

RNA was isolated from anthers and pistils from flower buds of 3-4 mm (1), 5-6 mm (2) or 10+ mm (3). Each lane contained 10 µg of total RNA and the membrane was hybridised with ^{32}P-labelled *Solanum tuberosum* flavonol synthase cDNA (FLS), *Solanum tuberosum* chalcone synthase cDNA (CHS), *Solanum tuberosum* flavanone-3-hydroxylase cDNA (F3H) (see Materials and Methods), and *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) as a control for loading.

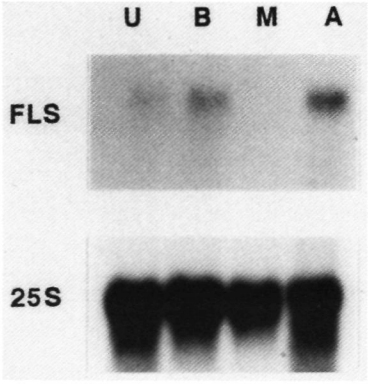

Figure 6 Northern blot analysis of developmental expression of *fls* in pollen

RNA was isolated from pollen at the following stages of developmental: U, unicellular pollen (5-6 mm buds), B, bicellular pollen (7-8 mm buds) and M, complete mature pollen (10+ mm buds). RNA of mature anthers (A) was used as a positive control. Each lane contained 10 μg of total RNA and the membrane was hybridised with ^{32}P-labelled stfls cDNA. For loading control, *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) was used.

stage it had increased drastically. This is in contrast to anthers of maize where the *fls* gene is not developmentally expressed (Deboo *et al.*, 1995). In pistils, the amount of fls transcripts did not change during further development, but in anthers the transcript level decreased. Chs transcripts were detectable in pistils of 3-4 mm buds, but in anthers they appear in the 5-6 mm stage. In both organs the transcripts are most abundant at maturity. The *f3h* gene was already expressed in both organs of the 3-4 mm flower bud, but the higher expression was in the anthers. During pistil development, *f3h* expression increased until the stage of 5-6 mm buds and had almost completely vanished in mature pistils and anthers. These data demonstrate that the *chs* gene, in anthers of the 5-6 mm stage, is the last of the three genes to be expressed which suggests that CHS can probably be the rate-limiting enzyme at the start of flavonol production in potato anthers.

Because flavonols were present in mature pollen but no fls transcripts were detected (Figure 4A), we investigated the possibility that the *fls* gene is expressed during earlier developmental stages. In pollen we measured the accumulation of fls transcripts at three developmental stages, unicellular (5-6 mm flower buds), bicellular (7-8 mm flower buds) and mature pollen (10+ mm flowers), and complete, mature anthers. The *fls* gene is transiently expressed during pollen development (Figure 6). At the unicellular and the bicellular stage fls transcripts accumulated, but fls transcripts were absent at the mature pollen stage.

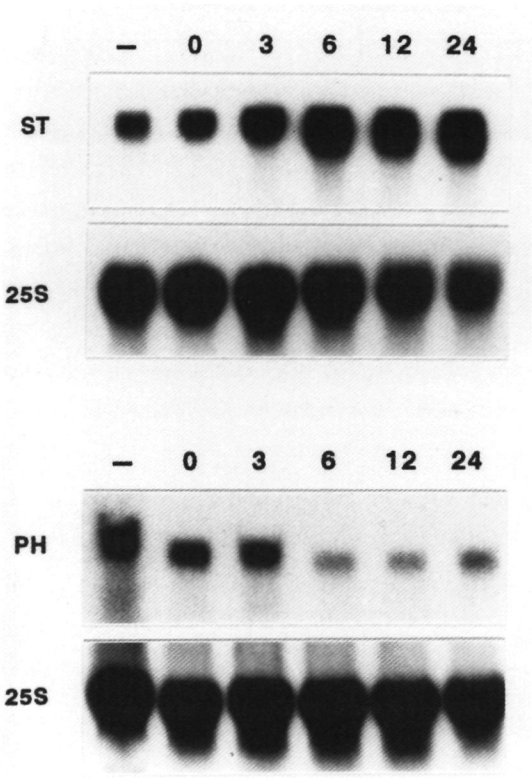

Figure 7 *Fls* gene expression during pollen tube growth in the pistil

RNA was isolated from pistils of *S. tuberosum* (ST) and *P. hybrida* (PH) 0, 3, 6, 12 or 24 hours after pollination. Unpollinated pistils (-) were used as a control. Each lane contained 10 µg of total RNA and the membrane was hybridised with ^{32}P-labelled stfls cDNA. For loading control, *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) was used.

Fls *gene expression during pollen tube growth*

The flavonol content of potato pistils did not change after pollination, unlike in *Petunia* pistils where the kaempferol content increased more than threefold (Figure 2B). We monitored the *fls* gene expression in pistils during the pollination process in both species (Figure 7). In pollinated pistils of potato, transcripts of the *fls* gene accumulated from 3 h to at least 24 h after pollination. At 3 h after pollination, the pollen just started to germinate and only a few pollen tubes were penetrating the pistil. In *Petunia*, the level of *fls* transcripts decreased from 3 h onwards after pollination. In this species, almost all the pollen had germinated 3 h after pollination and the tubes were growing through the stigmatic part of the pistil. These results underline that only the onset of gene expression may mark the beginning of accumulation of flavonols. If the compounds are already there, the stability of the enzymes and the instability of

the product may obscure any relation between transcript level and flavonol content.

Discussion

We have quantified flavonol accumulation during anther development and during pollen tube growth in the pistil, and related the accumulation pattern to the developmental expression of genes encoding enzymes of the flavonoid biosynthetic pathway. A flavonol synthase cDNA clone of *S. tuberosum* was isolated and characterised. The expression of this gene and two other genes, *chs* and *f3h*, was investigated. Flavonol accumulation started when the *chs* gene was transcribed in anthers and the products of the *chs* gene are probably regulating flavonol production in potato anthers. Fls transcripts are only present in unicellular and early bicellular pollen, but not in mature pollen. The accumulation patterns of flavonols in anthers and pollinated pistils were at no point in development correlated with *fls* or *f3h* gene expression.

The isolated potato flavonol synthase cDNA clone shows 85% amino acid identity with the *Petunia* fls cDNA clone (Holton *et al.*, 1993). This high homology together with the presence of two conserved dioxygenase domains strongly indicates that stfls encodes a potato flavonol synthase. The *fls* gene was expressed in anthers, pistils, petals and ovaries, parts of the flower where flavonols normally are accumulating (Davies *et al.*, 1993; Holton *et al.*, 1993; Koes *et al.*, 1990; Pollak *et al.*, 1993; Ylstra, 1995). The temporal expression pattern of the *fls* gene in whole flower buds of potato correlates well with the pattern of FLS enzyme activity in developing flower buds of *Dianthus caryophyllus* and *Petunia* (Forkmann *et al.*, 1985; Stich *et al.*, 1992).

Our results confirm that kaempferol is the most abundant flavonol in anthers of potato and *Petunia* (Ylstra *et al.*, 1994). In anthers of potato, all flavonols are present in a conjugated form, as has been described for *Petunia* and *A. thaliana* (Pollak *et al.*, 1993; Burbulis *et al.*, 1996). In potato anthers, flavonols start to accumulate at the unicellular stage of pollen development till the stage of maturity. Flavonol accumulation starts exactly at the same moment as in anthers of maize, *Petunia* and *Tulipa* (Deboo *et al.*, 1995; Kleinehollenhorst *et*

al, 1982, Pollak *et al*, 1993) The amount of flavonols in mature anthers of potato is comparable to the flavonol content in maize anthers (Deboo *et al*, 1995), but only half the amount as in *Petunia* anthers (W H Reijnen, unpublished results)

Of the three genes encoding enzymes of the flavonoid biosynthetic pathway investigated, only chs transcripts start to accumulate late enough to coincide with the start of flavonol accumulation in anthers F3h and fls transcripts are already present earlier in development The *chs* gene product, therefore, is most probably responsible for regulating flavonol biosynthesis in anthers of potato Flavonol and CHS enzyme accumulation also start at the same time in *Petunia* anthers (Pollak *et al*, 1993) But, in the maize it is *f3h* gene expression that coincides with the start of flavonol accumulation, which indicates that F3H probably regulates flavonol production in maize anthers (Deboo *et al*, 1995) This suggests a different regulation mechanism for flavonol biosynthesis in potato and *Petunia* anthers as compared with maize

Fls transcripts accumulate in uni- and bicellular pollen, but are no longer present in mature pollen These data open the possibility that flavonols can be produced in developing pollen and thus challenge the earlier contention that the tapetum is exclusively responsible for the presence of flavonols in pollen (Beerhues *et al*, 1989, Pollak *et al*, 1995, Vogt *et al*, 1995) Promoter activity of flavonoid genes, CHS transcripts and CHI enzyme activity have been established in developing pollen (Hauffe *et al*, 1993, Herdt *et al*, 1978, Ohl *et al*, 1990, Shen *et al*, 1992, Van Tunen *et al*, 1990) However, the presence of transcripts of the genes encoding all the enzymes of the flavonol biosynthetic pathway is necessary but not sufficient Further studies should reveal whether the corresponding enzyme activities follow the levels of gene expression

Enhanced flavonol accumulation upon pollen tube growth in the pistil is not a universal phenomenon Pistils of potato and maize do not respond to pollen tube growth in this respect (Pollak *et al*, 1995) Only pistils of *Petunia* respond to pollen tube growth by increasing the flavonol content (Vogt *et al*, 1994) Fls transcript accumulation during pollen tube growth in the pistil is also completely different in potato and *Petunia*, two members of the same family A comparison between potato and *Petunia* is further complicated by the fact that presence of flavonols may not be a prerequisite for functional pollen tube growth in potato The flavonol-deficient *A thaliana* tt4 mutant exhibits normal pollen tube growth and seed set (Shirley *et al*,

1995; Ylstra, 1995; Burbulis *et al.*, 1996). If flavonols are required for pollen viability in potato is not clear, and has to be proven by the analysis of flavonol-deficient potato plants.

This study suggests that CHS regulates the flavonol production in anthers of potato. Definite proof for the regulation by the first enzyme of the pathway should come from experiments in which flavonol accumulation is related to the activity of the enzymes involved in biosynthesis, because enzymes may show great differences in stability and different steps may be 'rate-limiting' at different developmental stages.

Acknowledgements

We thank Dr J-H. Jeon (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea) for providing the *Solanum tuberosum* chs-1a clone, Dr C. Tonelli (Università degli studi di Milano, Milano, Italy) for providing the *Vitis vinifera* f3h clone, Theo Geurts and Gerard van der Weerden for culturing the plants and René Theunissen for technical assistance. Critical manuscript editing was done by Dr. A.F. Croes. This work was supported by the EC Bridge programme BIOT 900172.

Materials and Methods

Plant material

Dihaploid *Solanum tuberosum* plants were grown in climate chambers at 20°C at a 16-hour day length. Development of *S. tuberosum* flowers was divided into four subsequent stages. Flower buds of 1-2 mm are characterised by a closed green bud and contain all the developmental pollen stages up to the tetrad stage, 3-4 mm flower buds are closed green buds with anthers containing tetrads, 5-6 mm flower buds are closed but the anthers are turning yellow and the pollen is in the unicellular stage. Flower buds of 7-8 mm are partly opened and

the petals and anthers are coloured and contain bicellular pollen. Flower buds over 10 mm correspond to flowers at anthesis (10+ mm) with completely developed and mature pollen. *Petunia hybrida* cv. W115 was grown under greenhouse conditions. Pollinations were carried out with mature pollen collected from anthers at anthesis and applied to the stigma of flowers with anthers just before anthesis. Pistils (without the ovary) were collected after different post-pollination times as indicated. All tissues were frozen in liquid nitrogen and stored at -80°C. Pollen at various developmental stages were isolated according to Schrauwen *et al.* (1990).

Nucleic acid methods

Isolation of plasmid DNA, subcloning and restriction analysis were performed using standard procedures (Sambrook *et al.*, 1989). Total RNA of the various plant tissues was isolated using the method of Frankis and Mascarenhas (1980) with slight modifications (Goldberg et al., 1981). The same procedure was used to extract high molecular-weight DNA from young leaves except that LiCl precipitations were omitted. The gel blot analyses were performed as described by Van Eldik *et al.* (1995). Northern blots were hybridised at 55°C and washed in 0.5x SSC; 0.1% SDS. For the detection of the various transcripts the chs-1a cDNA from *S. tuberosum* (Jeon *et al.*, 1996), the complete stfls cDNA and the cloned stf3h PCR fragment of *S. tuberosum* were used.

DNA sequencing

DNA sequencing was performed essentially by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) using the T7 DNA polymerase sequencing system of Pharmacia. The subclones of stfls, used for DNA sequence determination, were generated using an Erase-a-base kit (Promega). Both nucleotide and deduced protein sequences were analysed using the University of Wisconsin Computer Group programmes (Devereux *et al.*, 1984).

Molecular cloning of the STF3H PCR fragment

For Northern blot experiments, a part of the coding region of the *S. tuberosum* f3h mRNA was isolated. Two degenerated primers (F3H1 = 5'-CTCI(A/C)G(A/G)TGGCC(A/C/G)GA(C/T)

AA(A/G)CC-3'; F3H2 = 5'-GTTCACACA(C/G/T)GC(C/T)TG(A/G)TG(A/G)TC-3') were designed spanning a 330 nt fragment in the coding region of *f3h* as calculated from the *Vitis vinifera* f3h sequence (Sparvoli *et al.*, 1994). First-strand cDNA synthesis was done according to Kawasaki *et al.* (1990) on 10 µg of total RNA from mature *S. tuberosum* pistils. For the PCR reaction 4 µl of the reverse transcriptase reaction mixture was used. The PCR programme contained 40 cycles of 1 min 94°C, 2 min 55°C and 3 min 72°C. The PCR programme included an initial cycle with an extended denaturation at 95°C (10 min) and a final cycle with a prolonged extension at 72°C (10 min). The resulting PCR fragment hybridised to the f3h cDNA (Sparvoli *et al.*, 1994) of grape, was cloned into the pCRII vector using the TA cloning kit (Invitrogen) and subsequently sequenced.

Isolation of the S. tuberosum *flavonol synthase cDNA clone*

A cDNA library of cross-pollinated pistils (Van Eldik *et al.*, 1995) was screened using the cold-plaque method as described by Hodge *et al.* (1991) (see chapter 4). One of the isolated cDNA clones showed homology to the *P. hybrida* fls cDNA clone (Holton *et al.*, 1993).

Detection of flavonols

Extraction of tissue samples was performed by soaking tissues overnight in two volumes (w/v) 96% ethanol at -20°C. The tissues were crushed at room temperature, centrifuged, and the supernatant was hydrolysed in 2M HCl (1:1) at 60°C for 30 min. One half volume of 96% ethanol was added afterwards (Pollak *et al.*, 1993). Quantitative HPLC analysis of the flavonol content in fresh anthers and pistils was performed using the SMART system (Pharmacia). Chromatographic conditions were: 2 min isocratic in 10% acetonitrile/85% water/5% acetic acid followed by a linear gradient upto 70% acetonitrile/25% water/5% acetic acid in 15 min; flow rate 250 µl/min on a Sephasil C_{18}, 5 µm SC 2,1/10 column (Pharmacia). Peaks were detected at 365 nm. Kaempferol and quercetin were used as standards (ICN Biomedicals). The presented data are means of at least two separate tissue samples.

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CHAPTER 6

An isoflavone reductase-like gene is expressed upon touch and pollen tube growth in pistils of *Solanum tuberosum*

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Abstract

Successful sexual reproduction relies on a strong support of the pistil by delivering gene products to create an environment suitable for pollen tube growth. These compounds are either produced before pollination or specifically formed during the interactions between pistil and pollen tubes. In this chapter the pollination enhanced expression of the *cp100* gene in pistils of *Solanum tuberosum* is described. Analysis of temporal gene expression revealed enhanced *cp100* expression already one hour after pollination. Increased expression of the *cp100* gene was also established after touching the stigma and, because of increased levels of cp100 transcripts in the style, not restricted to the site of touch. The CP100 protein shows similarity to isoflavone reductase (IFR), an enzyme involved in the production of isoflavonoid phytoalexins in leguminous plants. Furthermore, it forms with other IFR-like proteins a new group of plant proteins.

Introduction

Sexual reproduction in flowering plants involves a series of interactions between pollen or pollen tubes, and pistil (Knox, 1984). These interactions start when pollen grains land on the stigma, adhere and germinate, and continue when pollen tubes grow through the style towards the ovary where double fertilisation takes place (Esau, 1977)

The various functions of the pistil, during the interactions with the pollen or growing pollen tubes, include recognition, guidance, nourishment and protection. Recognition involves the ability of the pistil to discriminate between different types of pollen it receives and to determine whether it will accept or reject the pollen (Knox, 1984) This process may be interspecific (incongruity), preventing pollen from other species to germinate and to form functional pollen tubes, or intraspecific (self-incompatibility), in case the pistil recognises self-pollen (Linskens, 1981, Sims, 1993)

Guidance is provided by the pistil when the pollen tubes are growing from the stigma

towards the ovules The morphology of the stigma, transmitting tissue and ovary determines the direction of pollen tube growth (Gasser and Robinson-Beers, 1993, Heslop-Harrison *et al*, 1985, Hulskamp *et al*, 1995) Chemotropism determines the direction of growth of pollen tubes *in vitro*, but there is only limited evidence for the presence of chemotropic substances guiding the pollen tubes from the stigma to the ovary (Cheung *et al*, 1995, Lord and Sanders, 1992, Mascarenhas, 1993, Willemse *et al*, 1995, Wu *et al*, 1995)

The pistil also produces nourishing compounds for the growing pollen tubes These extracellular compounds are present in the stigmatic exudate and in the stigma itself (Cresti *et al*, 1986, Kandasamy and Kristen, 1987, Konar and Linskens, 1966, Mackenzie *et al*, 1990), excreted by the cells of the transmitting tissue (Cheung *et al*, 1995, Herrero and Arbeloa, 1989, Herrero and Dickinson, 1979, Kroh *et al*, 1970, Labarca and Loewus, 1973, Lind *et al*, 1996, Wu *et al*, 1995), and by the ovary (Willemse and Franssen-Verheijen, 1988)

Protection against pathogens is required because the pistil is wounded due to the penetration of non-aseptic pollen tubes The production of defence molecules is expected during the interactions of the pistil with the growing pollen tubes However, such molecules may be more effective if present before pollination providing an active defence mechanism (Atkinson *et al*, 1993, Constable and Brisson, 1995, Gu *et al*, 1992, Karunanandaa *et al*, 1994, Leung, 1992, Lotan *et al*, 1989, Ori *et al*, 1990, Sessa and Fluhr, 1995)

These different functions of the pistil require pollen tube growth promoting gene products which are produced during pistil development before pollination or specifically formed after pollination by the interactions between pistil and pollen tubes Despite the role of the pistil in promoting pollen tube growth, no genes specifically expressed upon pollination have been isolated, although some pistil genes are described which show a modulated expression upon pollen tube growth (Goldman *et al*, 1992, O'Neill *et al*, 1993, Royo *et al*, 1996, chapter 5, Van Eldik *et al*, 1996, Wang *et al*, 1993)

We have used a molecular approach to identify genes that exhibit modulated expression patterns upon pollination, and to study the development and specialised functions of the pistil during interactions with pollen tubes as they grow towards the ovary To achieve these goals, a cDNA library of pollinated pistils of *Solanum tuberosum* was screened and several genes expressed predominantly, or exclusively in the pistil were isolated The characterisation and

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   1 CAAAGCGATTTACCAGTTTTAGAAATCAATGGCCGGAAAAAGTAAGATTCTGTTCATCGG
   1                             M  A  G  K  S  K  I  L  F  I  G

  61 CGGTACTGGTTACATTGGAAAGTTCATCGTCGAAGCTAGTGCTAAAGCTGGCCACGATAC
  12 G  T  G  Y  I  G  K  F  I  V  E  A  S  A  K  A  G  H  D  T

 121 ATTTGTCTTGGTTAGAGAATCCACTCTTTCCAATCCTACCAAAACCAAACTCATCGATAC
  32 F  V  L  V  R  E  S  T  L  S  N  P  T  K  T  K  L  I  D  T

 181 TTTCAAAAGTTTTGGCGTCACCTTCGTCCATGGAGATTTATATGATCATGAGAGTTTGGT
  52  F  K  S  F  G  V  T  F  V  H  G  D  L  Y  D  H  E  S  L  V

 241 GAAGGCGATAAAACAAGTGGATGTTGTGATTTCTACAGTAGGTCATGCCCTTTTAGCTGA
  72  K  A  I  K  Q  V  D  V  V  I  S  T  V  G  H  A  L  L  A  D

 301 TCAGGTCAAGCTAATTGCTGCTATCAAAGAAGCTGGCAATGTCAAGAGATTCTTTCCCTC
  92  Q  V  K  L  I  A  A  I  K  E  A  G  N  V  K  R  F  F  P  S

 361 TGAATTTGGGAACGATGTAGATCGTGTCCATGCTGTCGAGCCTGCTAAAGCAGCATTTAA
 112  E  F  G  N  D  V  D  R  V  H  A  V  E  P  A  K  A  A  F  N

 421 TACTAAAGCACAAATTCGCCGTGTTGTTGAGGCTGAAGGAATACCATTCACTTATGTGGC
 132  T  K  A  Q  I  R  R  V  V  E  A  E  G  I  P  F  T  Y  V  A

 481 CACCTTCTTTTTTGCTGGTTATTCTCTTCCAAATTTGGCACAGCCTGGAGCTGCAGGTCC
 152  T  F  F  F  A  G  Y  S  L  P  N  L  A  Q  P  G  A  A  G  P

 541 TCCCAACGACAAAGTTGTCATCTTAGGACATGGCAATACTAAAGCTGTTTTTAACAAGGA
 172  P  N  D  K  V  V  I  L  G  H  G  N  T  K  A  V  F  N  K  E

 601 AGAAGACATTGGCACCTATACCATAAATGCTGTCGATGATCCAAAAACACTTAACAAAAT
 192  E  D  I  G  T  Y  T  I  N  A  V  D  D  P  K  T  L  N  K  I

 661 CCTCTACATCAAGCCTCCACACAATATAATTACATTAAACGAGTTGGTATCCTTGTGGGA
 212  L  Y  I  K  P  P  H  N  I  I  T  L  N  E  L  V  S  L  W  E

 721 GAAGAAAACTGGAAAGAACCTTGAAAGATTATATGTACCAGAGGAACAAGTTCTCAAGAA
 232  K  K  T  G  K  N  L  E  R  L  Y  V  P  E  E  Q  V  L  K  N

 781 CATACAAGAAGCCTCGGTTCCAATGAATGTGGGATTATCGATCTATCACACTGCTTTTGT
 252  I  Q  E  A  S  V  P  M  N  V  G  L  S  I  Y  H  T  A  F  V

 841 GAAGGGTGACCACACTAATTTTGAAATTGAACCATCATTTGGAGTAGAGGCATCAGAGGT
 272  K  G  D  H  T  N  F  E  I  E  P  S  F  G  V  E  A  S  E  V

 901 TTATCCTGATGTTAAATATACGCCGATAGATGAGATTCTCAACCAGTATGTCTGAAAATG
 292  Y  P  D  V  K  Y  T  P  I  D  E  I  L  N  Q  Y  V

 961 TAGAGTTCCTTCTTATTAAGCATAAGGTTCTGATGCTCAATACCCAGCTCACGATTAGTG
1021 CTATTCGTATCAGCTTTCACCACTGTTTGTGAGTCCTCATC
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Figure 1 DNA sequence of the potato cp100 cDNA and the deduced amino acid sequence

The nucleotide sequence is numbered at the left (Accession number X92075). The sequence positions of the deduced amino acid sequence are relative to the starting methionine and indicated at the left. The potential NAD(P)H binding domain is underlined The start and stop codon are in boldface

expression of a *S. tuberosum* isoflavone reductase-like cDNA clone, designated cp100, is described in this chapter.

Results

Molecular characterisation of the potato cp100 clone

A cold-plaque screening (Hodge *et al.*, 1991) of a cDNA library from pollinated pistils of *Solanum tuberosum* resulted in the isolation of several cDNA clones representing pistil-expressed genes (see chapter 4). The characterisation of one of these clones, designated cp100, is described.

Sequence analysis of the cp100 cDNA clone revealed an open reading frame of 1061 nucleotides that encoded a protein of 308 amino acids with a predicted molecular weight of 34 kDa (Figure 1). The CP100 protein contained no cysteine. A potential NAD(P)H binding domain near the N-terminal end was localised from amino acid residues 7 and 37 (Figure 1) (Wierenga *et al.*, 1986).

A search through data bases revealed significant levels of amino acid identity of CP100 with four isoflavone reductase-like (IFR-like) sequences from *Arabidopsis* (69%), tobacco (62%), maize (59%) and white lupin (47%) (Babiychuk, Accession number Z49777; Attucci *et al.* 1996; Hibi *et al.*, 1994; Petrucco *et al.*, 1996). This group of IFR-like sequences displays homology to isoflavone reductases, a family of NADPH-dependent oxidoreductases from leguminous plants (Paiva *et al.*, 1991, 1994; Tiemann *et al.*, 1991). A multiple sequence alignment of CP100, the four other IFR-like sequences and the alfalfa isoflavone reductase protein is shown in Figure 2. These IFR-like proteins show, as compared to the IFR proteins from leguminous plants, a gap of 11 amino acids, which is located just behind the NAD(P)H domain and have an overall identity of 30%.

Genomic DNA was digested with EcoRI and BamHI and analysed by Southern blot hybridisation to estimate the number of cp100 sequences in the potato genome (Figure 3). Hybridisation with the complete cp100 cDNA as a probe resulted in four strong hybridising

bands which indicated that cp100 is present as a small gene family in the potato genome. Furthermore, a few faint hybridising bands are detected, indicating the presence of other less homologous sequences in the potato genome.

Expression of the cp100 *gene*

Analysis of the expression of the *cp100* gene in various potato tissues revealed the presence of cp100 transcripts in pistils, pollen, ovaries, anthers, petals, sepals, roots, leaves and stems (Figure 4A). So, the *cp100* gene was expressed in all generative and vegetative tissues at varying expression levels, although the expression levels in pistils and ovaries were substantially higher. The *cp100* gene was represented by a transcript of 1.1 kb.

Initial Northern blot analysis revealed that *cp100* gene expression was enhanced in pollinated pistils (see chapter 4). The up regulated expression upon pollination was further analysed. The expression of the *cp100* gene increased already 1 h after pollination and reached its maximum level of expression 6 h after pollination (Figure 4B). An increased level of cp100 transcripts was still present 72 h after pollination. This indicated that *cp100* gene expression continued after the tips of the pollen tubes had reached the ovary between 24 and 36 h after pollination. The transcript level of *cp100* was not modulated upon pollination with dead pollen.

Accumulation of cp100 transcripts in pistils after touching the stigma

During preliminary experiments on the temporal expression of the *cp100* gene during pollen tube growth, we loaded the hydrated pollen on the stigma with a paint brush. Surprisingly, we detected that just touching the stigma was sufficient to lead to accumulation of cp100 transcripts in the pistil. This indicated that the *cp100* gene can be induced by a mechanical stimulus that may preceed or be caused by the landing of the pollen. To determine the duration of the effect of mechanical stimulation on *cp100* gene expression, we isolated RNA from pistils 3, 6 and 24 h after touching the stigma with the brush and probed it with cp100 cDNA (Figure 5A). Six hours after touching the stigma, the level of cp100 transcripts had sharply decreased and after 24 h, the amount of cp100 transcripts was back to the initial level.

To determine if the mechanical inducibility of the *cp100* gene is restricted to the site of touching, we separated stigma and style prior to RNA isolation. After touching the stigma with

```
                1                                                    50
S.tuberosum CP100  ..MAGKSKILFIGGTGYIGKFIVEASAKAGHDTFVLVRE.....
A.thaliana P3      .MATE-----V--------------------S--A----.....
N.tabacum A622     MVVSE-----I---------YL--T---S--P--A-I--.....
Z.mays IFL         .MASE-----VV-----L-RHV-A---RL--P-SA---D.....
L.albus LA48590    ...MG---V-VV-----V-RR--K--LEH--E--I-Q-P.....
M.sativa IFRAFL1   ..MATEN---IL-P--A--RH--W--I---NP-YA---KTPGNVNKPKLI
                         * *  * **  *   *  *   *  *  * *

                51                                                   100
S.tuberosum CP100  STLSNPTKTKLIDTFKSFGVTFVHGDLYDHESLVKAIKQVDVVISTVGHA
A.thaliana P3      A---D-V-G-TVQS--DL---IL----N--------------------SM
N.tabacum A622     ---K--E-S--------Y---LLF--ISNQ---L--------------GQ
Z.mays IRL         TAP-D-A-AA-LKS-QDA---LLK-----QA---S-V-GA-----VL-SM
L.albus LA48590    EIGLDIE-LQILLS--KQ-AIL-EASFS--K---D---L-----C-MSGV
M.sativa IFRAFL1   TAANPE--EE---NYQSL--ILLE--IN---T---------I--CAA-RL
                         *         *             *  * *  * **

                101                                                  150
S.tuberosum CP100  L.....LADQVKLIAAIKEAGNVKRFFPSEFGNDVD.RVHAVEPAKAAFN
A.thaliana P3      Q.....-L--TK--S----------L-----V---.-TS------S--A
N.tabacum A622     Q.....FT---NI-K-------I---L-----F---.HAR-I---ASL-A
Z.mays IRL         Q.....I---SR--D----------------L---.-TGI-----SILG
L.albus LA48590    HFRSHN-LT-L--VE---D---I---L-----M-PALMG--L--GRVT-D
M.sativa IFRAFL1   -.....IE----I-K---------K-------L---.-HD----VRQV-E
                         *     *** *** * * ***** *        **

                151                                                  200
S.tuberosum CP100  TKAQIRRVVEAEGIPFTYVATFFFAGYSLPNLAQ..PGAAGPPNDKVVIL
A.thaliana P3      G-I----TI------Y--AV-GC-G--Y--T-V-FE--LTS--R---T--
N.tabacum A622     L-VR---MI------Y---ICNW--D-F----G-LE..AKT--R-----F
Z.mays IRL         A-VG---AT--AG--Y--AVAG-----G--KVG-VL..-P---A--A-V-
L.albus LA48590    E-MTV-KAI-EAN-----ISANC----FAG--S-MKTLL..--R---LLY
M.sativa IFRAFL1   E-AS----I----V-Y--LCCHA-T--F-R----LD..-TD--R------
                    *   *   *    * **     *  *      *       ** **

                201                                                  250
S.tuberosum CP100  GHGNTKAVFNKEEDIGTYTINAVDDPKTLNKILYIKPPHNIITLNELVSL
A.thaliana P3      -D--A---I------AA---K-----R----------SN-TLSM--I-T-
N.tabacum A622     -D--P--IYV-----A----E-----R----T-HMR--A--LSF--I---
Z.mays IRL         -D-D-----VE-G--A---VL-A---RAE--V------A-TLSH---L--
L.albus LA48590    -D--V-P-YMD-D-VA----KTI---R----TV-LR--E--L-HK--IEK
M.sativa IFRAFL1   -D--V-GAYVT-A----F--R-AN--N----AVH-RL-K-YL-Q--VIA-
                   * *  *     * *    *     **   **        *     *

                251                                                  300
S.tuberosum CP100  WEKKTGKNLERLYVPEEQVLKNIQEASVPMNVGLSIYHTAFVKGDHTNFE
A.thaliana P3      ----I--S--KTHL----L--S---SPI-I--V---N-AV--N--.--IS
N.tabacum A622     --D-I--T--K--LS--DI-QIV--GPL-LRTN-A-C-SV--N--SA---
Z.mays IRL         -------TFR-E-----A--KQ---SPI-L-II-A-G-A---R-EQ-G--
L.albus LA48590    --ELI--Q--KNSIS-KDF-STLKGLDFASQ--VGHFYHI-YE-CL----
M.sativa IFRAFL1   ----I--T--KT--S------D---S-F-H-YL-AL--SQQI---.AVY-
                   **   **        *   *                       *
```

```
                    301                             329
S tuberosum CP100   IEPSFGVEASEVYPDVKYFPIDEILNQYV 308
A thaliana P3       ----------L-------SV--Y-SYFA 310
N tabacum A622      VQ-PT----T-L--K----TV--FY-KF- 310
Z mays IRL          -D-AK--D---L-------TV--Y--RFL 309
L albus LA48590     -GEN -E----L--E-N--RM-QY-KV-- 312
M sativa IFRAFL1    -D-AKDI----A----T--TA--Y---F- 318
                          * * ** * **  *
```

Figure 2 Alignment of the deduced protein sequence of CP100 with IFR-like sequences and alfalfa IFR
The deduced IFR-like protein sequences of *Arabidopsis* (Babiychuk, Accession number Z49777), tobacco (Hibi *et al*, 1994), maize (Petrucco *et al*, 1996) and lupin (Attucci *et al*, 1996) and the IFR sequence of alfalfa (Paiva *et al*, 1991) are aligned using the PILEUP programme of the University of Wisconsin Computer Group (Devereux *et al*, 1984) Sequence positions of each sequence are indicated at the top The length of the proteins is indicated at the end of the amino acid sequence Amino acids identical to those in CP100 are indicated by dashes Dots represent a gap Amino acids that are identical between CP100, alfalfa IFR and all IFR-like proteins are marked by an asterisk

a paint brush, the *cp100* gene was found not only to be induced in the stigma, but also in the style (Figure 5B)

Discussion

For a better understanding of the role of the pistil during pollen tube growth, it is important to identify genes expressed upon pollination and to establish the function of the corresponding gene products in pollinated pistils We have isolated a cDNA, cp100, with a corresponding gene expression pattern up regulated in pistils upon pollen tube growth and, much more transiently, by touching the stigma This cDNA clone shows homology with various isoflavone reductase-like sequences

The predicted CP100 protein shows homology to the enzyme isoflavone reductase, present in leguminous plants (Paiva *et al*, 1991, 1994, Tiemann *et al*, 1991) This enzyme is involved in the production of isoflavonoid phytoalexins which accumulate in response to pathogen attack (Dixon *et al*, 1995) Since isoflavonoids are not synthesised in the solanaceous family,

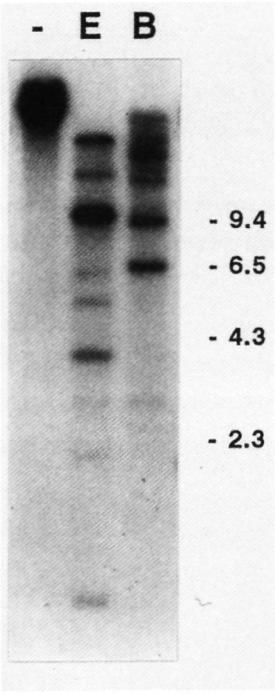

Figure 3 Southern blot analysis of genomic DNA
Total genomic DNA (20 µg) was digested with EcoRI and BamHI, and fractionated on an agarose gel, blotted onto a nylon membrane, and hybridised with ^{32}P-labelled cp100 cDNA insert. Molecular weight markers (kb) are indicated at the right.
-, undigested genomic DNA; E, *Eco* RI; B, *Bam* HI.

potato is not expected to have an isoflavone reductase gene (Harborne *et al.*, 1988). The CP100 protein is more similar to a small family of IFR-like plant proteins (Figure 2). This new family of plants proteins displays a gap of 11 amino acids as compared with the IFR proteins from leguminous plants. Drews *et al.* (1992) described the expression of another homologous IFR-like gene from tobacco, which is 68% identical to our CP100 protein (Drews and Goldberg, personal communication). Additional evidence, for the occurrence of an IFR-like family, is obtained by the absence of *in vitro* isoflavone reductase enzyme activity of the IFL and A622 IFR-like proteins (Hibi *et al.*, 1994; Petrucco *et al.*, 1996).

Petunia and *Datura* DNA both give a positive hybridisation signal when probed with cp100 cDNA and a pistil-expressed *Petunia* cp100 homologous cDNA clone has been isolated. This indicates that the family of IFR-like sequences is also present in other solanaceous plant species (Van Eldik and Van Herpen, unpublished results).

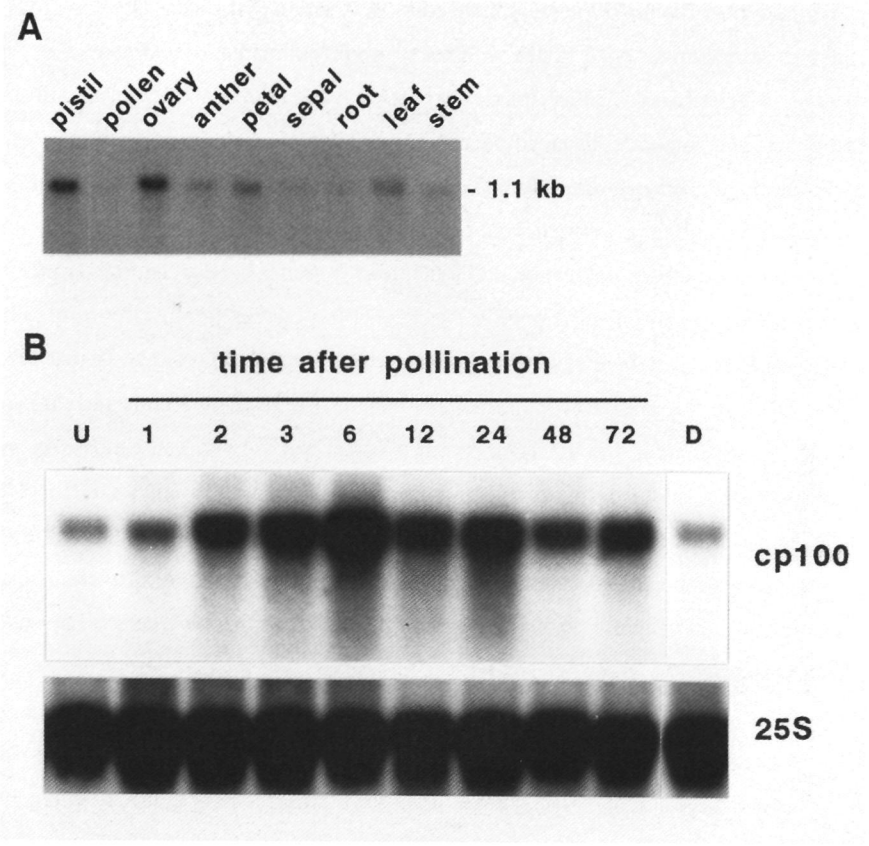

Figure 4 Northern blot analysis of *cp100* gene expression

A. Expression of the *cp100* gene in different potato organs. The size (kb) of the hybridising band is indicated. B. Expression of the *cp100* gene in pollinated pistils. Pistils were pollinated with hydrated pollen and collected after various time intervals (h). Unpollinated pistils and pistils pollinated with dead pollen were used as controls. Total RNA (10 μg per lane) was isolated, fractionated by agarose/formaldehyde gel electrophoresis, blotted onto a nylon membrane, and hybridised with ^{32}P-labelled cp100 cDNA insert. For loading control, *Nicotiana tabacum* 25S ribosomal rRNA cDNA (25S) was used. U, unpollinated pistils; D, pistils pollinated with dead pollen.

The expression of the *cp100* gene is induced by pollination and touch. Besides *cp100*, the expression of some other pistil genes is induced upon pollination (Goldman *et al.*, 1992; O'Neill *et al.*, 1993; chapter 5, Van Eldik *et al.*, 1996; Wang *et al.*, 1993), and a pistil-specific retrotransposon-like sequence linked to the S-locus of *Nicotiana alata* is expressed upon both pollination and touch (Royo *et al.*, 1996). Touch-induced gene expression is also described for genes of the calmodulin family (Braam and Davies, 1990; Takezawa *et al.*, 1995).

Within potato pistils both pollination and touch lead, within 3 h, to an enormous increase of the cp100 transcript level. The difference in duration of this increased transcript level, 6 h after touch as compared to 72 h in case of pollination (Fig. 4B and 5A), suggests that not only pollination but also the presence of the pollen tubes in the pistil is a constant stimulus for increased *cp100* expression. Due to the penetration of the non-aseptic pollen tubes, the pistil is wounded and, as a stress response, should be able to produce compounds involved in protecting the pistil against pathogens. Because pollination and touch, both situations of stress, result in the production of ethylene, this hormone could be a possible intermediate involved in increasing *cp100* gene expression (De Jaegher *et al.*, 1987; Jaffe *et al.*, 1985; Larsen *et al.*, 1995; O'Neill *et al.*, 1993; Telewski and Jaffe, 1986).

At present, a general function for the family of IFR-like proteins can not be established, because the expression patterns of the IFR-like genes, including *cp100*, are quite diverse. However, further research will include the analysis of transposon-tagged IFR-like mutant *Petunia* plants to define the function of the *cp100* gene products in the pistil upon pollen tube growth and touch, which can contribute to the understanding of how these processes function in relation to reproduction.

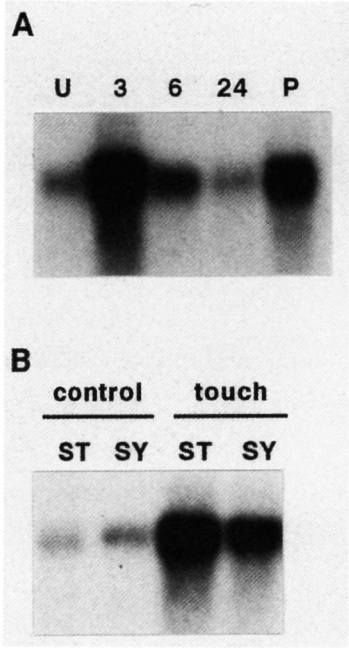

Figure 5 Touch-induced *cp100* gene expression

A. Expression of the *cp100* gene in pistils after touching the stigma with a paint brush, analysed after various time intervals (h). Pistils 24 h after pollination were used as control. B. Expression of the *cp100* gene in the stigma and in the style 3 h after touching the stigma with a paint brush. Untreated stigmas and styles were used as control. Total RNA (10 μg per lane) was isolated, fractionated by agarose/formaldehyde gel electrophoresis, blotted onto a nylon membrane, and hybridised with ^{32}P-labelled cp100 cDNA insert. P, pollinated pistils; ST, stigma; SY, style; U, unpollinated pistils.

Acknowledgement

We thank Theo Geurts and Gerard van der Weerden for culturing the plants. Critical manuscript editing was done by Dr. A.F. Croes. This work was supported by the EC Bridge programme BIOT 900172.

Materials and Methods

Plant material

Potato plants (*Solanum tuberosum*) were grown in climate chambers at 20°C under a light/dark regime of 16/8 h. Characteristics of plants were described elsewhere (chapter 2, Van

Eldik *et al.*, 1995) Pollinations were carried out with mature pollen collected from anthers at anthesis and applied to the stigma of flowers with anthers just before anthesis Dead pollen was obtained by irradiation of mature potato pollen with an ENRAF Roentgen apparatus equipped with an OEG-60 X-ray tube (Machlett Laboratories, Inc) at 50 kV and 32 mA, at a target distance of 4.5 cm and an exposure of 3.9 x 10^4 Sv. For the mechanical stimulation of pistils we touched the stigmas with a paint brush.

Nucleic acid methods

Total RNA was isolated using the method of Frankis and Mascarenhas (1980) with slight modifications (Goldberg *et al.*, 1981). DNA was isolated from young potato leaves according to the same method except that LiCl precipitations were omitted. The gel blot analyses were performed as described in chapter 2. Isolation of plasmid DNA, subcloning and restriction analysis were performed using standard procedures (Sambrook *et al.*, 1989) Nucleotide sequence analysis was performed using the T7 DNA polymerase sequencing system of Pharmacia. Both nucleotide and deduced protein sequences were analysed using the University of Wisconsin Computer Group programmes (Devereux *et al.*, 1984)

Isolation of cold-plaque cDNA clones

A cDNA library of pollinated pistils (chapter 2, Van Eldik *et al.*, 1995) was screened using the cold-plaque method as described by Hodge *et al.* (1991) (see chapter 4). A ^{32}P-labelled single-stranded cDNA probe, prepared from poly(A)$^+$ mRNA of pollinated pistils, was used. This was the same material from which the cDNA library was constructed. Approximately 10 000 pfu were screened at 45°C in 4x SETS (1x SETS is 0.15 M NaCl, 0.02 M TRIS-HCl pH 7 8, 1 mM EDTA), 5x Denhardt's (1x Denhardt's is 0 02% Ficoll, 0.02% PVP and 0 02% BSA), 0.1% SDS and 75 μg/ml denatured herring sperm DNA. This resulted in the isolation of 120 not reacting (cold) plaques.

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CHAPTER 7

Summary

Summary

The primary goal of the research presented in this thesis was to identify gene transcripts, ultimately responsible for the formation of products accumulating in the pistil of *Solanum tuberosum* during the interaction with growing pollen tubes and to relate these products to the various functions of the pistil To undertake a molecular approach, a cDNA library from pollinated pistils of *S tuberosum* was constructed and screened by two different screening techniques, differential and cold-plaque screening (chapter 2 and 4) The use of both screening methods turned out to be very successful and resulted in the isolation of a number of cDNA clones corresponding to genes preferentially expressed in the pistil and described in chapter 2 to 6 The analysis of the sequences and comparison of the predicted proteins to other known proteins enabled me to relate several of the recombinant clones to specific functions of the pistil The expression patterns of the corresponding genes, both temporal and spatial, provided additional information for the proposed functions of the predicted proteins

Among the isolated cDNA clones were sts14 and cp34, of which the encoded proteins showed homology to pathogenesis-related and γ-thionin-like proteins They are assumed to play a role in protecting the pistil against pathogen attack (chapters 3 and 4) Two other cDNA clones, cp71 and cp100, are homologous to genes specifically expressed upon treatments with hormones or in stress situations, but have no direct relation with the various functions of the pistil (chapters 4 and 6) In relation to the nutritional role of the pistil, CP67, an alcohol dehydrogenase-like protein was detected (chapter 4) In addition, cDNA clones encoding new, undescribed sequences were isolated which further underlines the success of the screening procedures (chapters 2 and 4) Finally, a cDNA clone encoding flavonol synthase, the enzyme responsible for the production of pollen-tube-growth promoting flavonols, was detected in both anthers and pistils, the reproductive tissues of the flower (chapter 5) In the potato anther, not flavonol synthase, but chalcone synthase is the rate-limiting step in flavonol biosynthesis In pollinated pistils of potato, pollen tube growth increases the expression of *fls*, but this resulted not in an increase of the flavonol content (chapter 5)

Because of the interactions between growing pollen tubes and pistil, a specifically induced expression of pistil genes is expected During our research on transcripts accumulating in

pollinated pistils of *S. tuberosum* we described two cDNA clones, cp100 and fls. Both genes showed an increased expression upon pollen tube growth, although the transcripts were also present in unpollinated pistils (chapters 5 and 6). Additionally, the induced expression upon pollen tube growth of the *sth-11* gene encoding alcohol dehydrogenase is reported (chapter 4). Based on the current data, it can be concluded that the pistil is prepared for pollination and contains the transcripts necessary to support pollen tube growth. Upon pollination, only a modulated expression of those genes takes place.

CHAPTER 8

Samenvatting

Samenvatting

Sexuele voortplanting is ook voor planten een van de meest cruciale processen. Het proces van voortplanting stelt de plant in staat zich te vermenigvuldigen en te verspreiden, en is van essentieel belang voor het vergroten van de variatie binnen het soort. Gedurende de groei van een zaadplant wordt een speciale structuur gevormd, de bloem, waarin het sexuele voortplantingsproces plaatsvindt. De bloem bevat gespecialiseerde organen, de helmknop en de stamper, waarin respectievelijk de mannelijke (pollen) en vrouwelijke reproduktieve cellen gevormd worden. Het al dan niet succesvol verlopen van de sexuele voortplanting is gebaseerd op interacties tussen het pollen en de stamper. De stamper vervult tijdens deze interacties verscheidene functies: herkenning, geleiding, voeding en bescherming van het pollen/pollenbuis. Het grootste gedeelte van het huidige onderzoek betreffende de pollen-stamper interacties is tot nu toe gericht geweest op de herkenningsfunctie van de stamper en omvat onder andere de analyse van de S-locus genen van de zelf-inkompatibiliteitssystemen, systemen in de plant waardoor zelfbestuiving voorkomen wordt. Voor de andere functies van de stamper zijn inmiddels verscheidene soorten genprodukten beschreven, hoewel, ondanks de vermeende pollenbuisgroei stimulerende rol van de stamper, nog geen genen zijn geïsoleerd die specifiek tot expressie komen na bestuiving. De stamper genen met een veranderd expressiepatroon na bestuiving die tot nu toe beschreven zijn, zijn geïsoleerd na de screening van onbestoven stamper cDNA banken.

Het primaire doel van het onderzoek gepresenteerd in dit proefschrift was de identificatie van gentranscripten, uiteindelijk verantwoordelijk voor de vorming van produkten die ophopen in de stamper van *Solanum tuberosum* (aardappel) tijdens de interactie met groeiende pollenbuizen en het relateren van deze produkten aan de verscheidene functies van de stamper. Voor een moleculaire aanpak werd een cDNA bank gemaakt van bestoven stampers van *S. tuberosum* en vervolgens gescreend met twee verschillende screeningstechnieken, differentiële en koude-plaque screening (hoofdstuk 2 en 4). Het gebruik van beide screeningsmethoden bleek erg succesvol en resulteerde in de isolatie van een aantal cDNA klonen welke corresponderen met genen die voornamelijk tot expressie komen in de stamper. Dit is beschreven in hoofdstuk 2 tot 6. De analyse van de DNA sequenties en de vergelijking van de daaruit voorspelde eiwitten met andere, bekende eiwitten stelde me instaat de producten van de cDNA klonen te relateren aan de specifieke functies van de stamper.

Onder de geïsoleerde cDNA klonen waren sts14 en cp34, waarvan de voorspelde eiwitten homologie vertoonde met pathogeen gerelateerde en γ-thionine-achtige eiwitten. Deze eiwitten worden verondersteld een rol te spelen bij de bescherming van de stamper tegen het binnendringen van pathogenen (hoofstuk 3 en 4). Twee andere cDNA klonen, cp100 en cp71, zijn homoloog aan genen die specifiek tot expressie komen in stress situaties of na een behandeling met hormonen. Echter de producten van deze klonen kunnen niet gerangschikt worden onder één van de eerder genoemde functies van de stamper tijdens pollenbuisgroei (hoofdstuk 4 en 6). In relatie met de voedende rol van de stamper werd een alcohol dehydrogenase-achtig eiwit, CP67, gevonden (hoofdstuk 4). Bovendien werden cDNA klonen coderend voor nieuwe, tot nu toe nog niet beschreven eiwitten geïsoleerd wat het succes van de screeningsprocedures onderstreept (hoofdstuk 2 en 4). Tenslotte werden de transcripten van een cDNA kloon coderend voor flavonol synthase (FLS), het enzym verantwoordelijk voor de produktie van pollenbuisgroei stimulerende flavonolen, aangetoond in zowel de helmknoppen als in de stampers, de twee reproduktieve weefsels van de bloem (hoofdstuk 5). Tijdens de ontwikkeling van de helmknop van de aardappel is echter niet het flavonol synthase enzym, maar chalcone synthase het beperkende enzym in de flavonol biosynthese. In bestoven aardappel stampers stimuleert de pollenbuisgroei de expressie van het *fls* gen, maar dit resulteert niet in een toename van de flavonol inhoud (hoofdstuk 5).

Vanwege de interacties tussen de stamper en de groeiende pollenbuizen wordt een specifieke inductie van de expressie van stampergenen verwacht. In ons onderzoek naar de accumulatie van transcripten in bestoven stampers van *S. tuberosum* beschrijven we twee cDNA klonen, cp100 en fls. De corresponderende genen vertonen een toegenomen expressie na pollenbuisgroei, hoewel de transcripten ook aanwezig zijn in onbestoven stampers (hoofdstuk 5 en 6). Bovendien wordt de geïnduceerde expressie na pollenbuisgroei beschreven van het *sth-11* gen coderend voor alcohol dehydrogenase (hoofdstuk 4). Gebaseerd op de huidige gegevens kan geconcludeerd worden dat de stamper is geprepareerd voor de bestuiving en al de transcripten bevat die nodig zijn voor de ondersteuning van de pollenbuisgroei. Na bestuiving vindt alleen een verandering in de expressie van deze genen plaats.

Nawoord

Na al die jaren van experimenteren en schrijven wordt het tijd om iedereen te bedanken die bijgedragen heeft aan zowel het onderzoek zoals gepresenteerd in dit boekje als aan hetgeen er niet in staat! Al het begin is moeilijk, niet alleen om sommige soorten planten in bloei te krijgen maar ook om de planten gedurende het hele jaar in bloei te houden Hiervoor was het werk van de mensen van de kas onmisbaar. Zonder hun voortdurende aandacht voor mijn aardappelplanten was het onderzoek aan de bloemen nooit van de grond gekomen

De begeleiders van mijn AIO-onderzoek, Rinus, Jan S en George, hebben een uitstekende taak verricht. Voor hulp bij het labwerk was er in het begin gelukkig Koen W om me met alle moleculaire dingen op weg te helpen Maar ook de andere AIO's leverden op allerlei gebieden raad en daad In eerste instantie Randy, John, Margryt, Anneke, Ton, Harry en Twan, en later de nieuwe AIO's en post-docs Floyd, Koen v/d H , Marc, Leon, Barend, Wim V , Jaap, Ron, Jobien en opnieuw Koen W , allen bedankt! Van het vaste personeel bedank ik iedereen van zowel 'boven' als 'beneden' en in het bijzonder Wim Reijnen voor het flavonolen werk Natuurlijk hebben 'mijn' studenten Carolla, Wim V , Miriam, Rene en Pieter een onmisbare bijdrage geleverd aan de resultaten die de basis vormen van al deze hoofdstukken

Het leren schrijven van artikelen valt niet mee, maar met de hulp van Ton Croes is het toch nog heel aardig gelukt Titti, jou kneepjes in de wang waren zeer ondersteunend Zonder de altijd hulpvaardige José en Theo zouden veel dingen nooit zo snel geregeld zijn

Mijn mede SI-groepslid René Ruiter zal ik nooit vergeten Vanaf de allereerste dag van onze AIO-tijd hebben we veel gedeeld. de kamer, labs, oplossingen, gels, RNA/DNA, de computer Bovendien hebben we samen experimenten verzonnen, plantmateriaal verzameld, proeven gedaan, resultaten besproken, naar de kas gelopen, naar de nieuwe blauwe maan geweest en congressen bezocht, en elkaar op allerlei andere gebieden geholpen René, twee AIO's weten meer, kunnen meer, doen meer en komen verder dan één, bedankt!

Als laatste wil ik Tineke bedanken, gewoon voor alles.

Curriculum vitae

Gerben van Eldik werd geboren in Oosterhout (Gld.) op 11 november 1966. Van 1979 tot 1985 volgde hij de Atheneum-B opleiding aan het Stedelijk Scholen Gemeenschap Nijmegen. In 1985 begon hij met de studie Biologie aan de Katholieke Universiteit Nijmegen. De biologie opleiding werd afgerond met drie stages. De stage voor de toegepaste richting werd uitgevoerd op het toenmalige ITAL te Wageningen, onder begeleiding van Dr. Ch.H. Hänisch ten Cate. De hoofdvakstage werd ingevuld op de afdeling Moleculaire Plantenfysiologie (Prof. Dr. G.J. Wullems) onder begeleiding van Dr. Wim van der Krieken, en een bijvakstage werd gedaan op de afdeling Biochemie (Prof. Dr. H. Bloemendal) met als directe begeleider Dr. Francy van de Klundert. De studie Biologie werd in april 1991 afgesloten, waarna hij in dienst trad van de Katholieke Universiteit Nijmegen als Assistent in Opleiding (AIO) bij de vakgroep Experimentele Plantkunde onder leiding van professor George Wullems. In de voortplantingsgroep van Dr. Jan Schrauwen en Dr. Rinus van Herpen werd in het kader van een EC-Bridge programma onderzoek gedaan naar de genexpressie tijdens de interacties tussen pollenbuizen en stamper van aardappelbloemen. Gedurende de AIO aanstelling werd tweemaal bij de cursus Plantenfysiologie voor tweede jaars studenten geassisteerd, en werden vijf biologiestudenten begeleid gedurende hun stage op de vakgroep Experimentele Plantkunde. Verder werd deelgenomen aan de door de 'Research School Experimental Plant Sciences' georganiseerde Autumn school 'Molecular and Genetic Analysis of Plant Genomes' in Wageningen en tweemaal aan de EC-cursus 'Sexual Plant Reproduction' in Nijmegen, bij welke laatste actief meegewerkt is bij de organisatie en uitvoering van het praktikum. Congressen en wetenschappelijk bijeenkomsten op het gebied van moleculaire plantkunde, bloemontwikkeling en pollen-stamper interacties werden bezocht in Amsterdam, Wageningen, State College (USA), London (UK), Köln (Duitsland) en Lyon (Frankrijk). Voor de posterpresentatie op het congres in State College werd een 'Travel Award' toegekend. Van april tot september 1996 heeft hij gewerkt binnen de vakgroep Experimentele Plankunde als post-doc medewerker in de secundaire metabolietengroep van Dr. Ton Croes.

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